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.The influence of asthma on the mouse ability to control the intranasal infection by the bacteria Brucella melitensis

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L'influence de l'asthme sur la capacité de la souris à contrôler l'infection intranasale par la bactérie *Brucella melitensis*.

The influence of asthma on the mouse ability to control the intranasal infection by the bacteria *Brucella melitensis*.

Mémoire présenté pour l'obtention

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L'influence de l'asthme sur la capacité de la souris à contrôler l'infection intranasale par la bactérie Brucella melitensis.

POTEMBERG Georges

Résumé

Brucella spp. sont des bactéries pathogènes intracellulaires facultatives responsables de la brucellose, une zoonose mondiale provoquant l'avortement chez les animaux domestiques et une maladie chronique fébrile associée à des complications graves chez l'homme. La brucellose humaine est transmise principalement par la consommation de produits alimentaires contaminés ou par aérosols. Il n'existe actuellement aucun vaccin approuvé contre la brucellose humaine et la thérapie antibiotique est longue et coûteuse.

La furtivité semble la principale stratégie de *Brucella* pour échapper au système immunitaire et persister chez son hôte. Contrairement à d'autres agents responsables de maladies granulomateuses tel que *Mycobacterium tuberculosis*, l'agent de la tuberculose, *Brucella* est n'est pas capable de persister à des niveaux élevés dans le poumon suite à une infection intranasale (IN). Une étude antérieure suggère que les réponses immunitaires de type T_H1 et T_H17 sont impliquées dans l'élimination de *Brucella* dans le poumon.

L'asthme est une hypersensibilité du système immunitaire à des antigènes environnementaux dénommé allergènes. Elle se traduit par un forte inflammation du tissu pulmonaire induite par une réponse immune de type T_H2. Parmi les allergènes les plus courants figurent les allergènes d'acariens. L'incidence de l'asthme a considérablement augmenté dans le monde depuis les années 1970. Selon l'OMS, en 2011, près de 300 millions d'individus souffriraient de l'asthme. Afin d'identifier les paramètres affectant la réponse immunitaire contre *Brucella* chez la souris, nous avons utilisé un modèle de cross-pathologies impliquant l'asthme allergique induit par des acariens (House Dust Mite) comme pathologie chronique préexistante. L'asthme a été induit par des injections IN répétées d'extrait d'HDM plusieurs semaines avant l'infection IN par *Brucella melitensis*. Nous avons observé que l'asthme allergique induit par HDM compromettait nettement la capacité des souris à contrôler l'infection IN par *B. melitensis*. La charge bactérienne est significativement augmentée de plus de 10 fois dans les poumons de souris asthmatiques. L'asthme permet également une persistance plus longue de *Brucella* dans le poumon. Ce phénomène est strictement dépendant de la réponse immune T_H2 associé à l'asthme car il est absent dans les souris STAT-6 déficientes qui ne sont pas en mesure de développer ce type de réponse. Nos données préliminaires suggèrent que l'asthme n'inhiberait pas la réponse immunitaire T_H1 protectrice contre *Brucella* mais favoriserait la multiplication de *Brucella* dans les macrophages alvéolaires du poumon.

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Summary

Brucella spp. are facultative intracellular bacterial pathogens responsible for brucellosis, a worldwide zoonosis that causes abortion in domestic animals and a chronic febrile disease associated with serious complications in humans. The human brucellosis is mainly transmitted through the consumption of contaminated food or by the inhalation of aerosols. There is currently no approved vaccine against human brucellosis and antibiotic therapy is long and costly.
The furtiveness seems to be the main strategy for *Brucella* to evade the immune system and to persist in the host. In contrast to other causative agents of granulomatous diseases such as *Mycobacterium tuberculosis*, the agent of tuberculosis, *Brucella* is not able to persist at high levels in the lungs following an intranasal (IN) infection . A previous study suggest that T_H1 and T_H17 mediated immune response are implicated in the control of *Brucella* in the lungs.
Asthma is an hypersensibility of the immune system to environmental antigens called allergens. This disorder displays an important inflammatory state of the lungs tissue induced by a T_H2 immune response. Among the most frequent allergens there are the house dust mites allergens. The incidence of asthma has significantly increased worldwide since the 1970s. According to the WHO, in 2011, almost 300 million people were affected by asthma. In order to define the parameters affecting this protective immune control, we used a cross-pathology model implicating house dust mites (HDM)-induced allergic asthma as chronic bystander pathology. We induced asthma by repeated IN injections with HDM extract several weeks before the IN infection with *Brucella melitensis*. We demonstrated that the HDM-induced allergic asthma phenotype compromises the ability of mice to control the intranasal infection with *Brucella melitensis*. The *Brucella* burden was significantly augmented of more than 10 folds in the lungs of asthmatic mice. The asthma also increases the persistence of *Brucella* in the lungs for a longer time. This phenomenon is strictly dependent of allergic asthma-induced T_H2 environment as it is absent in STAT-6 deficient mice that are not able to develop a T_H2 response. Our preliminary data suggest that asthma does not inhibit the development of a protective T_H1 immune response but rather promotes the replication of *Brucella* inside the alveolar macrophages of the lungs.

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CONTENTS

List of abbreviations:

-/-	gene deficiency
aBCV	autophagic BCV
AHR	Airway Hyper Responsiveness
AM	Alveolar Macrophage
APC	Antigen Presenting Cell
BALT	Bronchus Associated Lymphoid Tissue
BCG	Bacillus of Calmette and Guerin
BCV	*Brucella* Containing Vacuole
BMDM	Bone Marrow Derived Macrophage
Br-LPS	*Brucella* LPS
BV	Blood Vessel
CA	Conducting Airways
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
ER	Endoplasmic Reticulum
FcεR	Fragment Fc Receptor of IgE
GATA	Globin Transcription Factor
HDM	House Dust Mite
Ig	Immunoglobin
IFNγ	Interferon γ or type 3 Interferon
IL	Interleukin
IN	Intranasal
IP	Intraperitoneal
LPS	Lipopolysaccharide
MDLN	Mediastinal Draining Lymph Node
MHC	Major Histocompatibility Complex
MtB	*Mycobacterium tuberculosis*
NALT	Nasal Associated Lymphoid Tissue
NET	Neutrophil Extracellular Trap
NK	Natural Killer
NO	Nitric Oxygen
Omp	Outer Membrane Protein
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
RSV	Respiratory Syncytial Virus
STAT	Signal Transducer and Activator of Transcription
T4SS	Type 4 Secretion System
TCR	T Cell Receptor
T_{FH}	Follicular Helper T lymphocytes
TGF-β	Tumor Growth Factor β
TNF-α	Tumor Necrosis Factor α
TH	T Helper lymphocyte
TLR	Toll Like Receptor
Treg	regulatory T lymphocyte
TSLP	Thymic Stromal Lymphoprotein
WT	Wild type

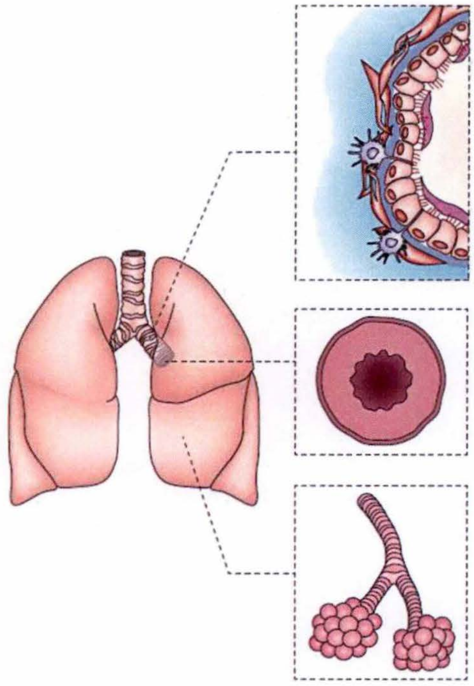


Figure 1: **General structure of the respiratory tract. Conducting airways leading to the alveolar sacs.**

The bronchial epithelium is composed of 4 layers (Mucosa, sub-mucosa, fibrocartilage layer and the interstitial layer). The mucosa is formed of ciliated cells and goblet cells. The fibrocartilage layer is composed of cartilage ring and smooth muscle. The interstitial layer comprises the lymphatics and blood vessels.
Bronchioles cross section. The bronchioles display continuous wall without cartilage and an important smooth muscle layer.
The small conducting airways have very small sections were the alveoli sacs are fixed. The alveoli sacs are exclusively formed of alveoli. (Edwards et al., 2012)

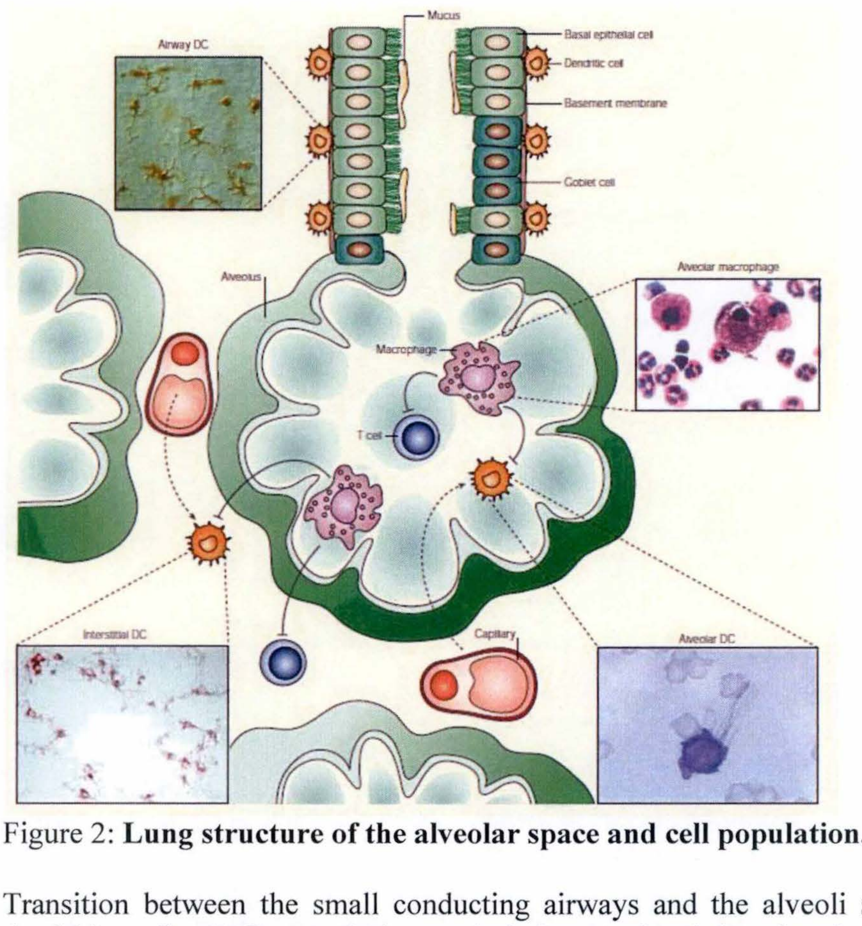


Figure 2: **Lung structure of the alveolar space and cell population.**

Transition between the small conducting airways and the alveoli sacs. The different dendritic cells (DC) population and their associated location in the lungs. Under homeostasis condition, the alveolar macrophages inhibit the activation of DCs and the function of T lymphocytes. (Lambrecht and Hammad, 2003)

INTRODUCTION

1st Part: Development of asthma model

Lung as a mucosal site

The lungs are an important mucosal site constantly exposed to pathogens, allergens and particles of the environment. In human, the volume of air inhaled by an individual each day is enormous, an average of 10,000 liters. That air contains all range of potential harmful entities for the body such as virus and bacteria (Williams, 2012). Most of human's pathogens penetrate the body through mucosal sites. To reach small airways, size matters. Small allergens and pathogens are more likely to spread throughout the lungs (Lloyd and Murdoch, 2010). Maintain the homeostasis in the lungs is a real challenge for the immune system. It needs to trigger the proper immune without doing any harm to the tissue. If the immune response is excessive, damages to the epithelium and so to the mucosal barrier could be done. Any damage could then open the way to pathogens and also to commensal microbial flora (termed microbiota) that are naturally present in the lungs (Mak et al., 2014).

The structure of the lung

The respiratory system is composed of two parts (figure 1): the upper airways also called the conducting system and the lower airways also called the gaseous exchange system (Williams, 2012; Young et al., 2006) .

The conducting airways comprise a succession of cavities (nose and mouth) converging both to the larynx to deliver the air inhaled to a single duct, the trachea. The trachea divides into two primary bronchi. These bronchi successively divide into smaller airways called bronchioles and at the last division step the airways become terminal bronchioles marking the end of the conducting airways. The main functions of the conducting system are to warm, moisture and clean the air before to reach the lower airways. These functions are assured by the mucosa of the conducting airways. This mucosa evolves progressively from a ciliated pseudostratified cylindric epithelium in the upper part of the trachea into a simple cubic cell epithelium at the end of the respiratory system (Figure 2). This progressive differentiation of the mucosa is to accommodate with the function of the two parts of the airways, with less mucus secretion in the gaseous exchange system. The conducting airways are also implicated in the regulation of the airflow by the smooth muscle layer lining the epithelium. This muscle layer reduces progressively up to disappear in the lower airways (Williams, 2012; Young et al., 2006).

The gaseous exchange system is the respiratory part of the lungs. It is responsible for gas exchanges between the air and the bloodstream. This system is composed of respiratory bronchioles, alveolar ducts and alveoli. Alveoli comprise dead end alveolar sacs opening on an open space. This structure maximizes the surface of epithelium in contact with air. The wall of the alveoli is made of a thin layer of epithelial cells of two types lying on a highly vascularized chorion (Williams, 2012; Young et al., 2006).

Box 2: The Immune System (Part 1)

The immune system is a mechanism under constant evolution. The objective of this system is to ensure the maintenance of the homeostasis of the body in different process (e.g. clearance of senescent cells) and contexts (e.g. infections). The predominant function of the immune system is to protect the integrity of the host against all range of pathogens such as bacteria, viruses, parasites, fungi but also against noxious substances like toxins. The immunity is composed by immune cells and soluble compounds presents in the whole body but also by physical barriers and lymphoid organs. The recognition of DAMPs (damage associated molecular pattern) or PAMPs is the first event that triggers an immune response. By recognition of molecular patterns, the immune system is able to distinguish self and non-self or even potential dangerous self. The interactions between humoral and cellular immune components, notably through cytokinic regulation, can lead to two types of immune responses: the innate and the adaptive. The complex interactions between the two responses is responsible for the "immunity" (Mak et al., 2014).

The innate immune response is characterized by a immediate response after the recognition by PRRs of evolutionary conserved patterns found in most of the microorganisms. This response will not be augmented in a challenge context as it displays a wide specificity and no long term memory. The innate immune system is mediated by both cellular and humoral responses but also by physical and chemical barriers. Natural antibodies and the activation of the complement can neutralize and opsonize the pathogen and also enhance the cell mediated immunity. After recognition, activated immune cells can induce the initiation of the inflammation through the secretion of cytokines but can also ensure the destruction of the pathogen by their effector functions such as phagocytosis (macrophages and neutrophils) or cytotoxicity (Natural Killer cells) depending on the cell properties (Williams, 2012).

The mucosal barrier

The mucosa is characterized by the production of mucus at the apex of the secreting cells of the epithelium. This mucus in the lumen of the conducting airways is produced by goblet cells. This mucus has an essential protective role in two ways. The mucus is the first physical barrier encountered by harmful agents. The composition of the mucus is the other contributor of this protection. The mucus is composed of mucin that keeps the epithelium moist and prevents bacterial adhesion to the epithelium. The active protection of mucus relies mainly on the presence of antimicrobial peptides and immunoglobulin (mostly IgA) (Box 1) to block both bacterial pathogens and commensals from entering. This mucus associated with ciliated cells forms the mucociliary elevator. The movement of the cilia pushes all the foreign bodies trapped by the mucus back to the aerodigestive intersection. This mucociliary apparatus is also responsible for the clearance of dust and dead cells (Mak et al., 2014; Williams, 2012).

> Box 1: IgA are poor inducers of immunity. By their dimeric form, the IgA are able to cross link several antigens to induce their agglutination and neutralization without eliciting an inflammatory response (Williams, 2012).

The immune system at mucosal site

The immune system at mucosal sites differs from the systemic immune system. Mucosal immunity is under constant regulation to avoid chronic inflammation and associated collateral damages. Mucosal immunity is a sensitive balance between tolerance of the exogenous antigens, symbiosis with commensal microbiota and maintenance of the control of pathogens. Homeostasis in the lungs is assured by a complex interplay of epithelial cells and resident immune cells. These resident immune cells include regulatory $CD4^+$ T cells (Tregs), macrophages and $\gamma\delta$ T cells (Lloyd and Murdoch, 2010).

Antigen tolerance and cell populations in the lung

The airway epithelial cells express some pattern recognition receptors (PRRs) at their membranes surface for recognition of pathogens. Among these PRRs, there is the Toll Like Receptors (TLRs) family. These receptors recognize pathogen associated molecular pattern (PAMPs) expressed by pathogens and once bound, they trigger rapid immune responses by the release of inflammatory cytokines and chemokines by the epithelial cells. As those epithelial cells encounter a large amount of bacteria, the expression of these TLRs is highly reduced even compared to other mucosa (Williams, 2012).

Alveolar macrophages are long life resident cells. In the major airways, this cell type resides in the mucus layer but they are distinct from alveolar macrophages that reside in the alveoli juxtaposed to alveolar epithelial cells. Alveolar macrophages are one of the most abundant immunocompetent cell type found in the alveoli. Their location in the lungs placed them in first line to encounter antigens and pathogens but alveolar macrophages have low respiratory burst (Hussell and Bell, 2014). Under certain circumstances like intracellular infections, alveolar macrophages can migrate to the lymph nodes (Archambaud et al., 2010). However, they have poor antigen presenting cells (APCs) function compared to dendritic cells (Holt et al., 2008; Jakubzick et al., 2006). To maintain homeostasis, alveolar macrophages display immunosuppressive properties to inhibit inflammation (Lauzon-Joset et al., 2014). Alveolar macrophages produce anti-inflammatory cytokines such as interleukin (IL)-10 and tumor growth factor (TGF)-β to target dendritic cells (DCs) and inhibit their activation (Holt et al., 1993).

Box 2: The Immune System (Part 2)

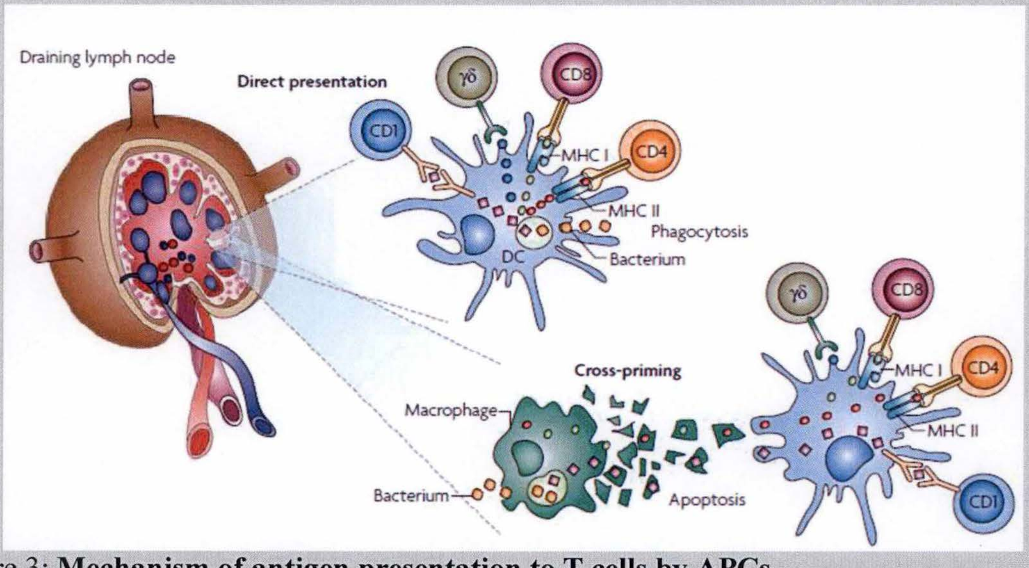


Figure 3: **Mechanism of antigen presentation to T cells by APCs.** (Kaufmann, 2007)

Antigen presenting cells (APCs) are at the interplay between innate and adaptive immunity. They are able to integrate all the signals received and to drive adaptive responses in lymphoid organs. Macrophages and dendritic cells (DC) are professional APCs. They can phagocyte pathogens and then process the antigens into peptides that will be presented at their major histocompatibility complex (MHC). The recognition of the MHC by the T cell receptor (TCR) of the naive T lymphocytes, co stimulatory ligands and cytokines expressed by the activated APCs, induce the maturation of the naive T cells into effector T cells. The antigen presented by MHC class I or II activate $CD8^+$ or $CD4^+$ T cells respectively. (Figure 3) $CD4^+$ T cells regulate and organize the immune response by their production of cytokines. $CD8^+$ T cells are also called cytotoxic T lymphocytes due to their ability to kill the targeted cells by the release of perforin and granzyme. After activation, these effector T cells proliferate and migrate to the infected tissue. At the same time, B cells are activated with the help of helper T (TH) cells, switch their Ig isotype production and become plasmocytes producing massive amounts of antibodies. When the infections is resolved and the homeostasis reset, only T and B memory lymphocytes persist. The memory lymphocytes are ready to respond directly and will induce an enhanced response if the antigen is encountered again (Kaufmann, 2007). Adaptive immune response is an effective response specific to the antigen but is delayed by the length of the procedure (Mak et al., 2014).

Insterstitial macrophages are a very heterogeneous cell populations distinct from the alveolar macrophages by their location in the lung and their phenotype (Bedoret et al., 2009). They are found in the wall of the airways underlying the epithelium (Young et al., 2006). This cell population is still not well described but it seems they have immunomodulatory properties. It has been shown that interstitial macrophages prevent DCs to induce allergic responses against innocuous allergens during the background TLR signaling (Bedoret et al., 2009).

Tregs mediate an important mechanism of tolerance to inhaled allergens. Tregs have been shown to reduce allergic inflammations by secretion of IL-10 (Akbari et al., 2002). Their immunosuppressive action is dependent on their localization as their action can also be mediated by cell-to-cell contact. These cells reside in the lung parenchyma but can be found in the airways and lymph nodes after allergen challenges (Lloyd and Murdoch, 2010).

γδT cells are unique resident cells of the lungs. Their γδ T cell receptors (TCRs) are antigen dependent but with a wide specificity similar to innate immune cells. γδ T cells have diverse properties being at the interface of the innate and adaptive immunity. Depending on the environment context and antigen recognition, these cells have paradoxal activities. In steady state conditions, they have a regulatory capacity for the maintenance of homeostasis. However, they can promote allergic responses to certain allergen expositions or inflammation during bacterial infections notably by their production of IFNγ and their cytotoxic activity (Lloyd and Murdoch, 2010; Vantourout and Hayday, 2013).

The lung immune responses

The immune system of the lungs must discriminate between the background signals (microbiota, allergens, etc.) and signal induced by pathogens. The respiratory system is equipped with a rich network of DCs. This network of sentinels constantly sample inhaled antigens. The DCs capture the antigens by cellular projections through the epithelium. They are able to recognize pathogen-associated molecular pattern (PAMPs) as well as damage-associated molecular patterns (DAMPs) by the collection of receptors present at their cell surface. The cytokinic environment induced by activated epithelial cells can also activate DCs. The epithelial cells can by this way, modulate the induction of the T helper immune response through the DCs (Lambrecht and Hammad, 2009).

Once activated, DCs can bypass the regulation of the Tregs and migrate to the draining lymph nodes of the lungs or to discrete clusters of immune cells underlying the epithelium. These clusters are called bronchus associated lymphoid tissue (BALT) or nasal associated lymphoid tissue (NALT) depending on their location. In the lymph nodes and in other secondary lymphoid organs, mature DCs have the potency to induce the activation of naive T cells and their proliferation. DCs mediate the cross talk between the innate and adaptive immunity. DCs initiate the adaptive immune responses and the development of a memory state. Activated lymphocytes then migrate back through efferent lymphatics to the tissue where the antigen was first encountered. This is achieved through tissue specific homing mechanism (Holt et al., 2008).

Box 3: The IFNγ, a potent cytokine in the T_H1

The IFNγ is a cytokine produced by both innate and adaptive immune cells. This cytokine play a central role in early defense against intracellular pathogens. Once the IFNγ binds its receptor, it activates downstream pathways involved in the regulation of the expression of T_H1 genes notably through STAT1. The IFNγ signaling pathway can enhance the antimicrobial activity of T_H1 effector cells and their secretion of T_H1 cytokines (Mak et al., 2014; Williams, 2012). The T_H1 response will be more deeply described in the next part.

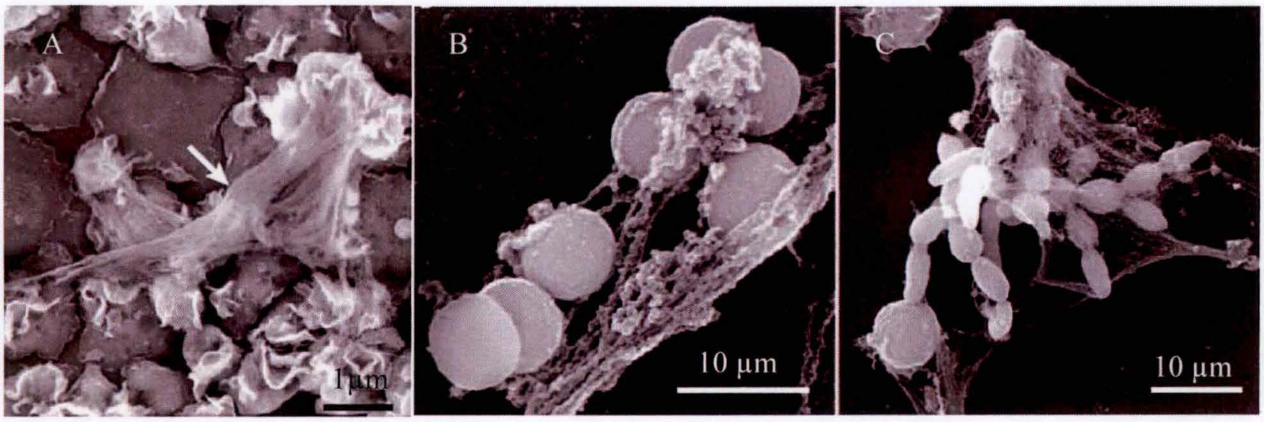


Figure 4: **Neutrophils undergoing NETosis**

A. The white arrow shows a neutrophil forming Neutrophil Extracellular Traps
B. The bacterium *Staphylococcus aureus* trapped in NETs.
C. The yeast *Candida albicans* trapped in NETS.
(Brinkmann and Zychlinsky, 2007)

The activation of other innate immune cells induces a dramatic switch in their functions and capacities depending of the type of signal. The activation of human alveolar macrophages polarizes them into different phenotype associated with very different functions. Classically activated alveolar macrophages (M1 macrophages) have enhanced capacity to phagocytosis and killing. These M1 macrophages induced by TLR ligands and interferon (IFN)-γ signals are polarized for a T Helper (T_H)-1 mediated response as they secrete pro-inflammatory cytokines such as IL-1, IL-12, IL-23 and Tumor Necrosis Factor (TNF)-α. Alternatively activated alveolar macrophages (M2a macrophages) participate to resolution of the inflammation and promote healing of the damaged tissue. These M2a macrophages are induced by IL-4 and IL-13 receptor via STAT-6 dependent signal and are polarized for a T_H2 response as they secrete pro-inflammatory cytokines. Alveolar macrophages are a plastic population; they can also reverse their phenotype (Holt et al., 2008; Hussell and Bell, 2014). Under inflammatory conditions, this cell population is renewed by the recruitment of monocytes (Holt et al., 2008).

In a pro-inflammatory context (T_H1 response) against bacterial pathogens, migration of monocytes goes with massive recruitment of neutrophils to ensure the clearance of bacteria (Williams, 2012). Through a size sensor mechanism, neutrophils can decide the more appropriate response to a pathogen. They will phagocyte the pathogen if it is small enough but if not, the neutrophil will rather undergo NETosis (Branzk et al., 2014). NETosis is a cell death program through which the neutrophil projects its chromatin contents outside the cell as a web along with antimicrobial substances to trap pathogens. These projections are called Neutrophil Extracellular Traps (NETs) (Fuchs et al., 2007) (Figure 4). This is a slow process compared to phagocytosis and engages the cell fate. This size sensor mechanism minimizes the damages induced but excessive neutrophils infiltration still leads to important tissue damages (Branzk et al., 2014).

This neutrophils chemotaxis could be also regulated by the IL-23 – IL-17 immune axis. In the lungs, the T_H17 seems to be implicated in the early control of bacterial infections but also in the pathogenesis of autoimmune diseases. IL-17 producing cells have a large plasticity in their range of functions (Gaffen et al., 2014). Early control of infectious disease by innate immune cells at mucosal sites such as the lung, can be mediated by the production of IL-17 notably by $\gamma\delta$T cells like in *Mycobacterium tuberculosis* infections (Peng et al., 2008). Myeloid cells such the neutrophils can also produce IL-17 after lipopolysaccharide (LPS) exposure in the lungs (Cua and Tato, 2010; Ferretti et al., 2003). In addition to the control of diverse microorganisms, T_H17 cells are implicated in the mucosal barrier integrity and in the resolution of tissue damages (Gaffen et al., 2014).

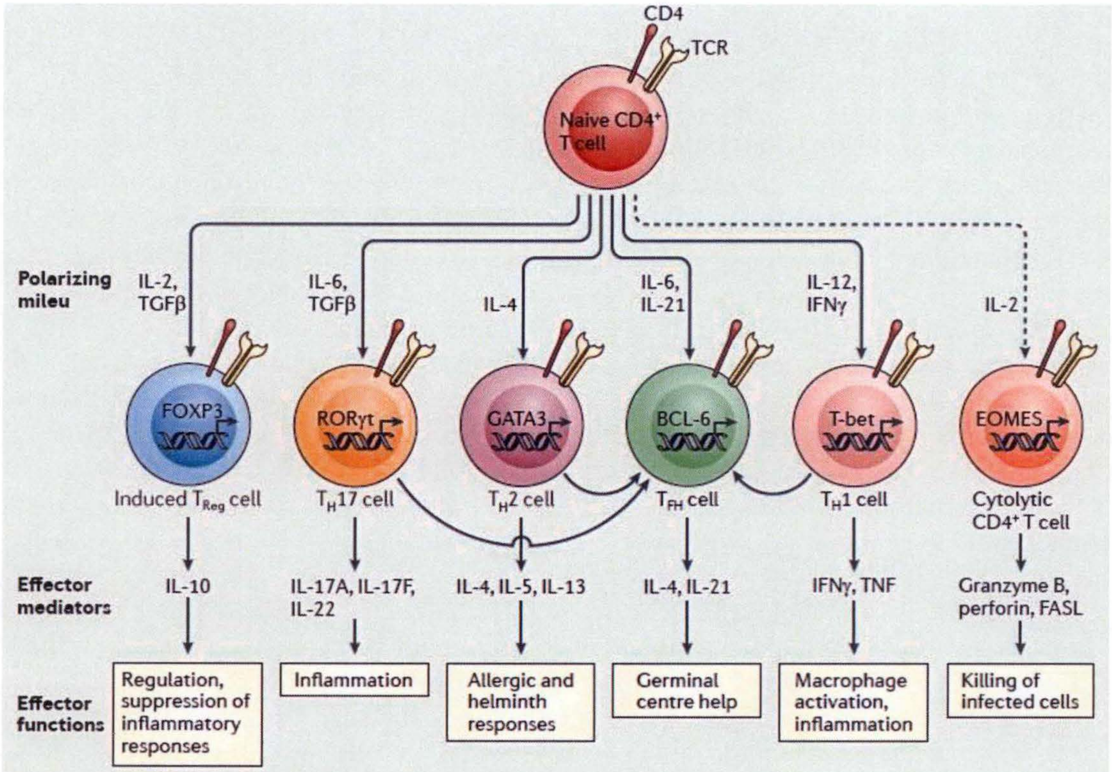


Figure 5: **The different types of T_H cells and associated responses**

Depending on the cytokinic environment, the naive CD4+ T lymphocytes polarized into a distinct type of T_H effector cells. The polarization has great importance as it will define its effectors and associated functions. The polarization is orchestrated by specific transcription factors.

IL, Interleukin, TCR, T cell receptor, TH, T helper lymphocytes, IFNγ, Interferon γ, TNF, Tumour necrosis factor.

(Swain et al., 2012)

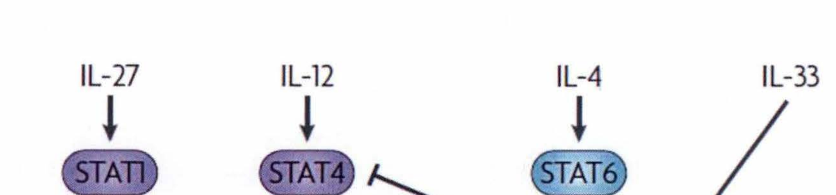


Figure 6: **The T_H1- T_H2 cross inhibition**

The relative cross inhibition between the T_H1 and the T_H2 immune responses is mediated through their respective transcription factors T-bet and GATA3. The T_H1 cells secretion of TH1 cytokines (IL-12 and IFNγ) is regulated by the transcription factor T-bet and induced by the IL-27 and IL-12. The T_H2 cells secretion of T_H1 cytokines (IL-4, IL-5 and IL-13) is regulated by the transcription factor GATA3 and induced by the IL-4 and IL-33.

GATA3, GATA binding protein 3, STAT, Signal Transducer and Activator of Transcription.

(Barnes, 2008)

2nd Part:

The T helper immune responses

As mentioned before, $CD4^+$ T helper (T_H) cells are generated during the crosstalk between activated DCs and naive $CD4^+$ T cells. This crosstalk induces the proliferation of activated T cells and their maturation into T helper cells. Depending on the different signals integrated by the DCs that are transmitted to the T cells, T cells can give rise to various subsets of T_H cells (figure 5). The nature of the pathogens often play a major role in the polarization of the T_H response (Swain et al., 2012). The T_H cells organize the different effectors to ensure the control of the infection and the survival of the host (Ouyang et al., 2008). The T_H cells have various functions that are responsible for the generation of cellular and humoral immune responses. They stimulate the production of antibody by the B cells and promote the generation cytotoxic T cells and associated memory (Diebold, 2008).

For a long time, the T_H1 and the T_H2 responses were the only T_H responses well characterized. The description of host's immunity against infectious agents and the explanation of the evasion mechanisms of these pathogens were based on the antagonism between these two responses (Mosmann and Coffman, 1989). It is now clear that it is far more complex than this paradigm. New subsets have been discovered and improve our understandings of the role of immunity in some pathologies. These new subsets includes the Treg cells, T follicular helper (T_{FH}), the T_H17, T_H22 and other T_H immune responses (Swain et al., 2012).

The T_H1 immune response

Virus and intracellular bacteria such as *Mycobacterium tuberculosis* (Potian et al., 2011) and intracellular protozoan parasites like *Leishmania major* (Sacks and Noben-Trauth, 2002) elicit in resistant individual the development of a T_H1 response to control the infection. The exposure of the naive T cells to particular cytokines such as IFN-γ and IL-12, induces the expression of the transcription factor t-bet. The expression of t-bet then polarizes the differentiation of naive T cells into T_H1 cells but also regulates negatively the transcription factors involved in the T_H2 pathway (Figure 6). These T_H1 cells produce large amount of pro inflammatory cytokines such as IFN-γ, which in turn initiate and emphasize the T_H1 immunity. IFN-γ impacts the effectors functions of both innate and adaptive cell types. This cytokine enhances the phagocytic activity of macrophages and their synthesis of nitric oxide (NO) species and reactive oxygen species (ROS). IFN-γ can also increase the expression of the major histocompatibility complex (MHC) class I or class II that will improve the cytotoxic activity of $CD8^+$ T lymphocytes to kill infected cells or improve the antigen presenting ability of APC (Williams, 2012). The IFN-γ is also a potent inducer for B cells isotype switching that will produce IgG2a and IgG3 (Swain et al., 2012).

The T_H2 immune response

The protective response against parasitic helminths such as *Nippostrongylus brasiliensis* (Chen et al., 2012), is mediated by the induction of a T_H2 response (Diebold, 2008). However, the process involved in the initiation of this T_H2 response and the subsequent maturation of the T_H2 cells are still unclear. Recent studies have highlighted the importance of the cytokines released by mucosal epithelium during exposure to innocuous allergens or during helminths infections (Palm et al., 2013). Helminths and allergens could trigger a T_H2 response through their protease activity and associated tissue damages (Donnelly et al., 2006; Palm et al., 2012). Under these conditions, the cytokines released by the injured epithelium such the IL-33 (Liew, 2012) together with the recognition of DAMPs could then initiate the development of the T_H2 response (Palm et al., 2013) (Figure 6). These results lead to reconsider the phenomenon of atopy[1] induced by innocuous allergens in some individual. These allergens through their potential activities could be regarded as toxic compounds. The allergic response then would be a defense mechanism against toxic substances (Profet, 1991). Recent studies sustain this hypothesis (Marichal et al., 2013; Palm et al., 2013). The T_H2 immune response and the IgE production induced after small exposition to honeybee or to viper venom, confer resistance to subsequent exposition to a lethal dose. The host is able to improve its resistance to toxins by the development of a T_H2 immunity.

The T_H2 cells by their secretion of IL-4 and other T_H2 cytokines like the IL-5 and IL-13, promote the IgE isotype switch of the B cells (Holgate, 2012). The production of specific IgE potentiates the activity of the mast cells. The exposition of the IgE armed mastocytes to the antigens then triggers the release of the effectors of the mast cells. The infectious agents covered by IgE have reduced adherence and specific IgE can neutralize some toxins produced by the pathogen (Diebold, 2008).

The organization of the T_H2 immunity is regulated by the $CD4^+$ T_H2 cells through their secretion of IL-4, IL-5 and IL-13. The cytokines induce the recruitment of effector cells such eosinophils, mast cells and basophils (Holgate, 2012). The most described of these cytokines is the IL-4. In human and mice, the IL-4 signal is regulated by the signal transducer and activator of transcription (STAT)-6. Through this pathway, the IL-4 has an important role in the induction of the transcriptional profile of the T_H2 cell effectors and in the maintenance of this phenotype (Elo et al., 2010). The IL-4 - STAT6 pathway is needed at the site where the T_H2 response take place to promote the effector mechanisms and the maintenance of the T_H2 response (Mathew et al., 2001; O'Shea et al., 2011) (Figure 6).

The T_H17 immune response

In an infectious context, time matters. Early onset of an appropriate response to the pathogens is sometimes crucial to achieve a complete clearance. The T_H17 immune response is characterized by a rapid and effective immunity to a wide range of extracellular pathogens such bacteria, fungi and parasites (Cua and Tato, 2010). Humans with deficiencies in this pathway are more susceptible to general infections of the lungs including fungal infections (e.g. *Candida albicans*) or bacterial infections (e.g. *Staphylococcus aureus*) (Gaffen et al., 2014). During pulmonary infection with *Klebsiella pneumoniae*, IL-17 producing T cells are necessary to efficiently control the bacteria (Ouyang et al., 2008). The T_H17 ensures its rapid

[1] Predisposed susceptibility to produce IgE in response to innocuous antigens (Edwards et al., 2012)

response mainly through the innate immune cells. The T_H17 cells are implicated in the recruitment of neutrophils, macrophages and NK cells to the site of infection which can lead to excessive inflammatory response (Cua and Tato, 2010). If this inflammation become chronic or in the case of a deregulation of the pathway, the T_H17 can be involved in the pathogenesis of autoimmune disorders such as psoriasis (Ouyang et al., 2008). The T_H17 immunity is expected to be a transient response inducing the development of a T_H1 or a T_H2 at later stage (Diebold, 2008).

The T_H1-T_H2 cross inhibition

The immune system is able to adapt its response to ensure the control of the infection and at the end, restore the homeostasis. To be effective, the polarization of the response has to take the nature of the pathogen, its life cycle and persistence in the host context into account (Muraille et al., 2014). First described by the Mosmann and Coffman's T_H1-T_H2 paradigm, the polarization of the immunity is the result of the cross inhibition between the T_H responses (Figure 6) (Mosmann and Coffman, 1989). This regulation is mediated by the main transcription factors and their associated STAT through the inhibition of expression of their opposite counterparts. In T_H1 cells, STAT4 is induced by the IL-12 signal and repress the STAT6 expression by epigenetic modifications but also induces the activation of t bet (O'Shea et al., 2011). T bet is a central factor of transcription promoting the T_H1 profile and downregulate GATA3, the transcription factor responsible for the T_H2 phenotype. On the other hand, T_H2 cells downregulate t bet by their expression of GATA3 (Barnes, 2008).

The cross-pathology concept

Some pathogens are able to skew the polarization of the immunity as an evasion mechanism to persist. Pathogens that have acquired immunomodulatory mechanisms manipulate the immune reactions to persist in the host. Such phenotype of the pathogens can lead to chronic pathologies (Muraille et al., 2014). People living in developing countries are often affected by multiple chronic infections. A chronic bystander infection is an important risk factors contributing to higher susceptibility to secondary infections (Stelekati and Wherry, 2012). These bystander infections alter the immune status of the tissue driving the immunity towards a certain type of response or another. The polarization of the immune cells such as macrophages, can delay their reaction time to a stimulus and reduces their capacity to control secondary infections under certain conditions. Constant inflammatory conditions lead to increased damages of the tissues and at mucosal sites, breaking the epithelial barrier and opening the way to new pathogens (Habibzay et al., 2013).

Example: *Haemophilus influenzae* and *Streptoccocus pneumoniae*

The 1918 influenza pandemic resulted in millions and millions of deaths caused by a severe pulmonary pathology. After clinical investigations, it came out that almost all the patients who suffered from pneumonia had bacterial complications. The predominant bacteria found in the patient's lungs were *Streptoccocus pneumoniae.* Nowadays, the presence of *S. pneumoniae* in patients with pneumonia is still linked with a worse outcome. This influenza strain caused severe injuries to the lung tissue and enhanced the availability of receptors needed for bacterial adherence. The T_H1 immune response induced against influenza virus through the IFNα may also impair the efficiency of the innate immune cells and in the course of infection, can deplete the pool of macrophages. This lack of macrophages enhances the susceptibility to secondary infection and in some cases, can induce massive recruitment of neutrophils exacerbating the inflammation but without any impact on the control of the infection (McCullers, 2014). This example of co-infection illustrates how bystander infection can increase the susceptibility to secondary infections by impeding the onset of the proper immune response.

Example: *Helicobacter pylori* and allergic asthma

Helicobacter pylori is an ancestral bacterial pathogen of the human gastric tract. This pathogen is associated with the induction of gastric ulcers and preneoplasic pathologies (Arnold et al., 2011). These last decades, the presence of this bacteria in the gastric microbiota decreased at a point of an almost disappearance in neonates (Banatvala et al., 1993). Following the hygiene hypothesis the reduction of the human microbiota could be linked with the increasing rates of atopic disorders such as allergic asthma (Blaser and Falkow, 2009; Strachan, 1989). Allergic asthma is characterized by an inflammatory state of the lungs under a TH2 response. A recent study showed that *H. pylori* by its immunomodulatory properties prevents the pathogenesis of allergic asthma in infected mice. This protection was provided by the infiltration of Tregs in the lungs induced by the infection localized in the stomach. The immunosuppressive activity of the Tregs inhibited the onset of the T_H2 response by the repression of the DCs maturation (Arnold et al., 2011). This study supports the hygiene hypothesis. The microbiota is involved in multiple physiological process of the host and chronic infections can be either beneficial or detrimental.

Choice of allergic asthma for cross pathology

As illustrated above, cross-pathology study model have raised increasing interest these last years. Used as a tool, co-infections models have the potency to unveil actors and mechanisms implicated in the immunity against pathogens (Stelekati and Wherry, 2012).

Asthma is a chronic pathology of the lung affecting more than 235 million people worldwide this includes developing countries where brucellosis is endemic. The prevalence of asthma and allergy has steadily increased over the last decades (Devereux, 2006; Pappas et al., 2006) (WHO, 2013). Asthma is also a pathology that can affect both human and animals (Bates et al., 2009). Asthma is a well described pathology and is still a field of research. Allergic asthma is characterized by a T_H2 immune response localized in the lung. Inflammatory disorders of the lung can impairs immunological responses leading to bacterial superinfections and worse outcome for the patients (Habibzay et al., 2013).

In this study, we used the cross pathology concept to evaluate the impact of allergic asthma on an intranasal infection with *Brucella melitensis* to discover the immunological mechanisms needed to control the infection in the mouse model.

The following parts of the introduction will describe the two pathologies of interest.

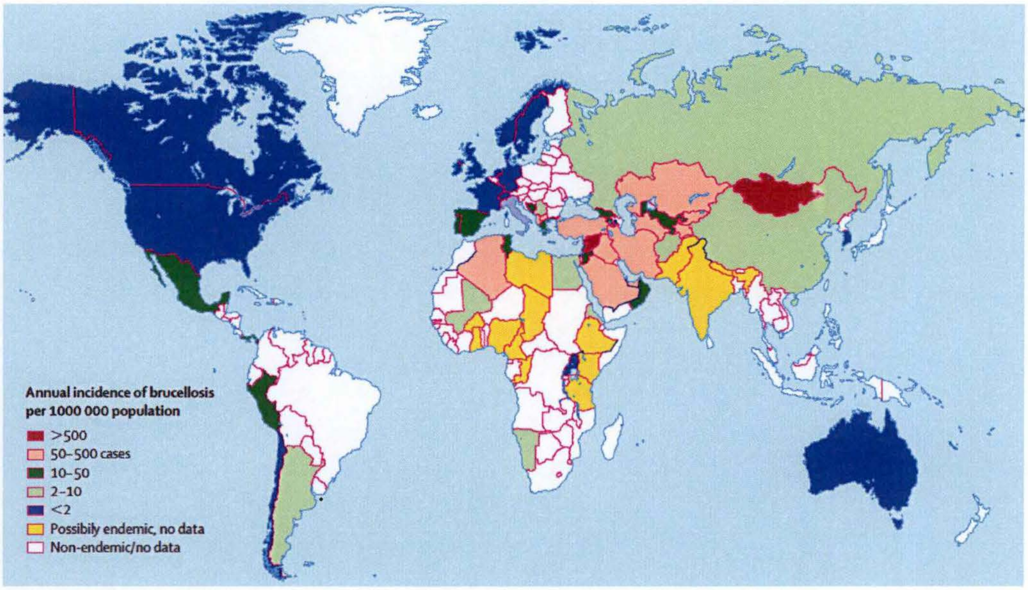


Figure 6: **World map of brucellosis incidence in 2006.**
(Pappas et al., 2006)

Species	Host preference	Zoonotic potential[a]
Brucella melitensis	Sheep, goat (*Ovis* spp. and *Capra* spp.)	High
Brucella abortus	Cattle (*Bos taurus* and *Bos indicus*)	Moderate
Brucella suis	Pig (*Sus scrofa*)	Moderate
Brucella canis	Dog (*Canis lupus familiaris*)	Mild
Brucella ceti	Dolphin, porpoise, whale (Cetacea)	Mild
Brucella pinnipedialis	Seal (Pinnipedia)	Mild
Brucella inopinata	Unknown	Mild
Brucella ovis	Sheep (*Ovis* spp.)	No reported infections
Brucella neotomae	Desert woodrat (*Neotoma lepida*)	No reported infections
Brucella microti	Common vole (*Microtus arvalis*)	No reported infections
Brucella sp. (baboon isolate)	Baboon (*Papio* spp.)	No reported infections

Table 1: ***Brucella* species and related host preference according their virulence to humans.**

Brucella melitensis, *Brucella abortus* and *Brucella suis* are the most virulent species for humans. *Brucella ceti*, *Brucella pinnipedialis* and *Brucella inopinata* human infections are less frequently reported.
(Atluri et al., 2011)

3^{rd} Part:

The brucellosis

The epidemiology

Brucellosis is the most common zoonosis worldwide (Pappas et al., 2006). Each year, more than 500,000 human cases are diagnosed. This infectious disease is endemic in different sparse regions of the world. Brucellosis is still endemic in regions such as the Mediterranean basin, Mexico and south America but have emerged and spread rapidly in central Asia and middle East (Figure 6). As brucellosis is a zoonosis, the incidence of human brucellosis is linked with the incidence of the disease in animals, its natural reservoir. Campaigns of eradication of the disease mainly rely on vaccination to decrease the prevalence and then on the elimination of the entire herd when an animal is infected. This way, western countries are now considered as brucellosis free (Pappas et al., 2006). However brucellosis can still re-emerge from the wildlife reservoirs for which the control is challenging. In low income countries, brucellosis is a huge economical burden as animal slaughter campaigns are costly (Martirosyan and Gorvel, 2013). For now there is two live attenuated vaccines: S19 (*B abortus*) and Rev1 (*B. melitensis*) but still no effective human vaccine available (Godfroid et al., 2011).

The pathology and the transmission:

Brucella species are facultative intracellular pathogens causing brucellosis in infected hosts. These Gram-negative bacteria are able to infect both humans and animals. The *Brucella* genus is composed of 11 different species. Each species is associated with preferential hosts ranging from wildlife animals such as whales (*B. ceti*), common vole (*B. microti*) and rodents (*B. neotomae*) to domestic animals like the dogs (*B. canis*) and farm animals such as swine (*B. suis*), cattle (*B. abortus*) and sheep (*B. melitensis*) (Godfroid et al., 2011) (Table 1). Brucellosis in animals results in abortions and sterility, induced by the colonization of the reproductive tract by *Brucella*. The horizontal transmission between naturals hosts is frequently the result of the consumption of contaminated milk, mating or the inhalation of aerosols released during the abortion. Humans are accidental hosts for *Brucella* as transmission between humans is anecdotic. The consumption of raw milk products is the leading cause of human infections. However, the airborne pathogen is able to survive in aerosols and once inhaled, *Brucella* can easily find its way through the human respiratory tract. Airborne and direct contact transmission are regarded as an occupational disease as it happens most often to veterinarians, farmers and butchers (Atluri et al., 2011). Among the *Brucella* species, six of them are pathogenic for humans but the most infectious one is *Brucella melitensis* (Godfroid et al., 2011). Human brucellosis is characterized by an undulant fever and weakness in the acute phase leading often to a misdiagnosis. If not treated by a combination of synergistic antibiotics by example a combination of Rifadine 600mg/Doxycycline 200mg a day for 6 weeks. Otherwise, the disease becomes chronic. The infection can then spread to the osteo-articular system, the heart or even the brain. The chronic phase is associated with increased morbidity, debilitations and life threatening complications (Xavier et al., 2010).

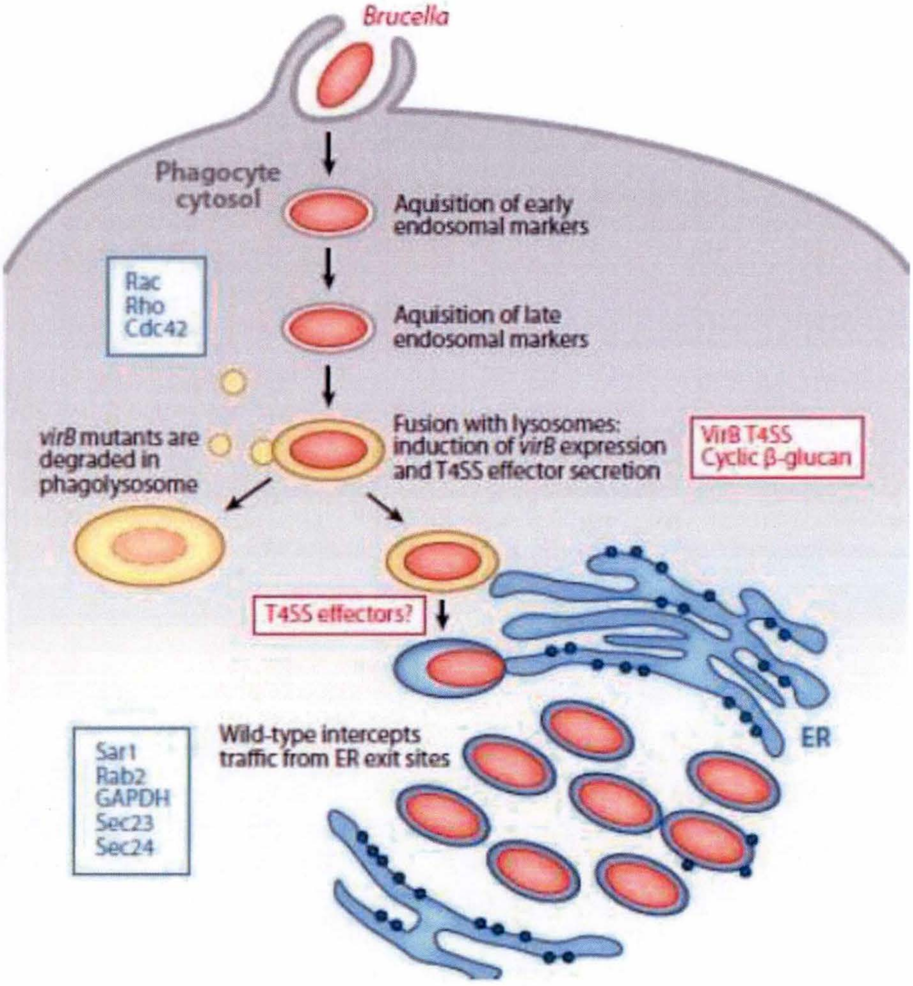


Figure 7: ***Brucella* intracellular lifecycle and implicated effectors.**

The intracellular lifecycle of *Brucella* in the endosomal system of a macrophage is mediated by both host cell components (written in blue) and *Brucella* factor (written in red). *Brucella* evades from the phagolysosome and reaches its replicative niche in the endoplasmic reticulum.
ER, endoplasmic reticulum, T4SS, Type 4 Seretion System
(Atluri et al., 2011)

Course of infection of *Brucella*

Little is known about the kinetic of *Brucella* dissemination following pulmonary infection. It has been shown that alveolar macrophages (AM) are the first cell type infected at 24h post infection. AMs are a replicative niche for *Brucella abortus* supporting its survival and replication. The first days of infection, *Brucella*, hiding in these cells, does not elicit inflammation. Infected AMs and some DCs carry out the infection to the draining lymph nodes of the lungs such the mediastinal draining lymph nodes (MDLN). In the MDLNs, *Brucella* carries on its replication in both AMs and DCs that have migrated from the lung (Archambaud et al., 2010). One week after the pulmonary infection with *Brucella melitensis,* there is a progressive dissemination to other organs such the spleen or the liver (Hanot Mambres et al., under submission). How this transmission is mediated is still unclear but one study has shown that *in vivo*, red blood cells can be infected (Vitry et al., 2014). Interestingly, the course of infection of *Brucella* in the lungs is only transient (Atluri et al., 2011). *Brucella* is rapidly cleared from both the lungs and the liver of mice. In the chronic phase *Brucella* will persist in the spleen and the osteo-articular system (Hanot Mambres et al., under submission) (Rajashekara et al., 2005).

The *Brucella* intracellular lifecycle

Brucella is a stealth pathogen able to survive and replicate in professional phagocytes. *Brucella* has developed many strategies to manipulate these cells and evade their bactericidal effects. The manipulation of the host starts even before the entry of *Brucella* in the cells. *Brucella* lowers the host recognition through the PRRs by modifications of its surface components (Martirosyan and Gorvel, 2013). The modified lipopolysaccharide (LPS) lipid A of *Brucella* has a reduced potency to be recognized by the TLR4 - MD2 complex and so to trigger an immune response (Conde-Álvarez et al., 2012) (Figure 8). The long length of the LPS O chain results in a steric hindrance that can prevent the action of some antibacterial substances such as the complement (Lapaque et al., 2006). The global lipid composition of the cell envelope is non canonical which reduces its recognition (Martirosyan and Gorvel, 2013).

Brucella involves a lot of systems and associated effectors to mediate its entry and lifecycle in the host cell. *Brucella* can bind to sialic acids at the surface of the cells to mediate its adhesion (Castañeda-Roldán et al., 2006). This process is maybe mediated trough adhesin(s) (Iannino et al., 2012). The entry inside the host cells is still unclear and depend on the nature of the target cell. The infection of macrophages can occur through different mechanisms such as the IgG opsonization or through lipid rafts regulated by the class A scavenger receptor and the cellular prion protein (Atluri et al., 2011).

Once inside the phagocyte, *Brucella* is in a vacuole called the *Brucella* containing vacuole (BCV) following at first, a normal traffic towards the phagolysosome. The BCV undergoes a transient interaction with early endosomes (Figure 7). Then the BCV interacts with late endosome and lysosomes which is associated with the acquisition of late endosomal markers such as LAMP1 and acidification of the vacuole respectively (Starr et al., 2008). The acidic conditions through the BvrR/BvrS system trigger the gene expression of the VirB operon resulting in the expression of the type 4 secretion system (T4SS) (Lacerda et al., 2013). This T4SS secretes and regulates a lot of effectors notably needed to evade complete fusion with the lysosomes and subsequent degradation. In this aim, the cyclic β-glucan, another virulence factor acts conjunctly with the T4SS (Arellano-Reynoso et al, 2005). The BCV is then redirected

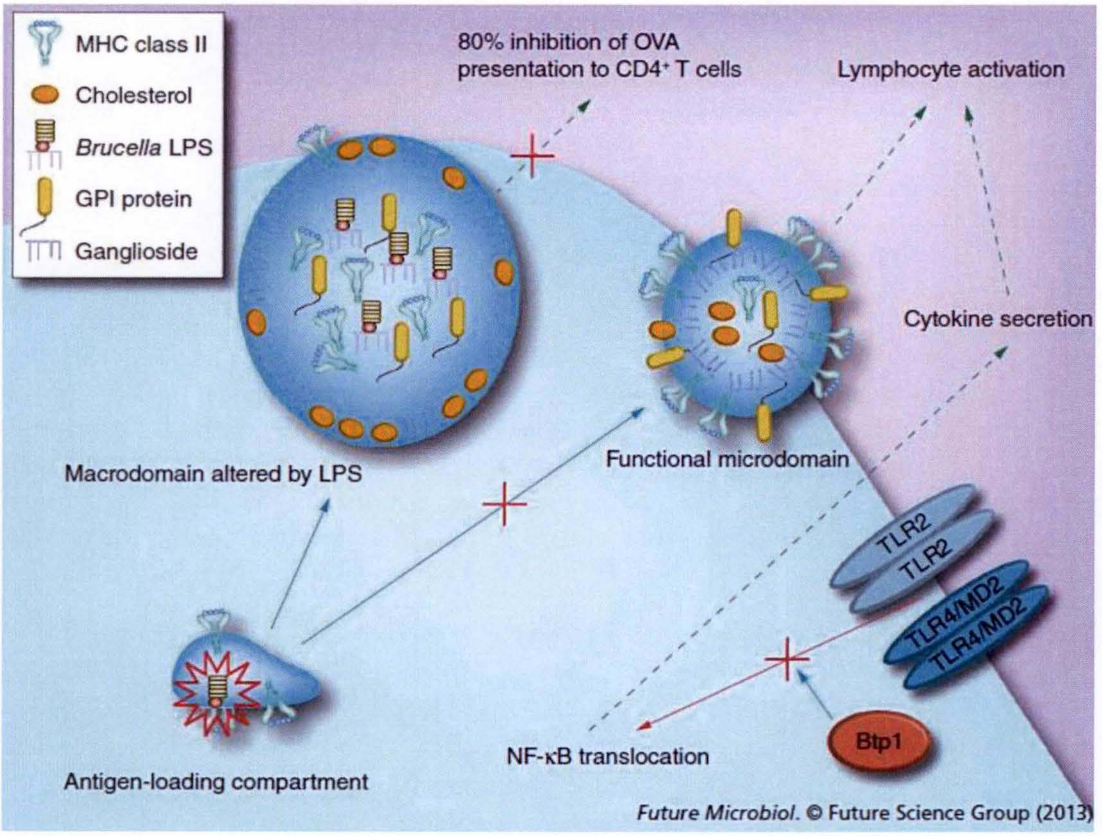


Figure 8: ***Brucella* evasion mechanisms and virulence effectors**

Brucella has developed many strategies to modulate both innate (blue arrows) and adaptive immune system (dashed black arrows). BtpB is a translocated effector, able to block the downstream signaling of the TLR4 and the TLR2. The unconventional structure of the *Br*-LPS forms inactive complexes with the MHCII called macrodomains. These complexes assemble during the processing of the antigen for the presentation. It results in less MHCII presentation which in turn, leads to a decreased activation of T lymphocytes.

TLR, Toll like receptor, MHC, Major Histocompatibility Complex, LPS, lipopolysaccharide

(Martirosyan and Gorvel, 2013)

towards the endoplasmic reticulum (ER). This is mediated by the tubulovesicular system of the golgi apparatus under the regulation of the Rab2 complex. Finally, the BCV reach the ER, the replication niche for *Brucella* (Brumell, 2012).

How *Brucella* disseminates from one cell to another is still unclear. In a recent study, a new type of BCV called autophagic BCV (aBCV) was observed at late stage infections of both Hela cells and bone marrow derived macrophages (BMDM) *in vitro*. This aBCV displaying a double membrane independently of the elongation system is thought to be mediated by a non canonical autophagy pathway. This autophagy pathway was associated *in vitro* with secondary dissemination. *Brucella* could manipulate the autophagy pathway of the host cell to mediate its spread to other cells (Starr et al., 2012).

The immune response against *Brucella*

<u>The innate immune response</u>

Brucella as a furtive bacteria, has evolved strategies to reduce the signals leading to the development of an immune response. *Brucella* can hide from the immune system thanks to its outer membrane composition and by the expression of effectors (Martirosyan and Gorvel, 2013) (Figure 8). The unconventional structure of the *Br*-LPS forms inactive complexes with the MHCII. These complexes assemble during the processing of the antigen for the presentation. It results in less MHCII presentation which in turn, leads to a decreased activation of T lymphocytes (Forestier et al., 1999). In addition, *Brucella spp.* have effector proteins such as the BtpB. BtpB is a translocated effector, able to block the downstream signaling of the TLR4 and the TLR2. These receptors recognize the *Brucella* LPS (*Br*-LPS) and lipoprotein respectively (Salcedo et al., 2013). Despite all its furtive properties, *Brucella* is still recognized by the immune system. The recognition of unmethylated CpG of the *Brucella* DNA by the TLR9 conjunctly with the activation of the TLR4 have been shown to be necessary to induce inflammatory cytokines. The Myd88 adaptor is implicated in the regulation of the downstream signal of the TLRs. Myd88 is crucial for the inflammatory DCs maturation from the monocytes (Copin et al., 2007).

After recognition by the innate immune cells, some mature DCs manage to establish the interaction between the innate and the adaptive immunity. The mature DCs secrete IL-12 and TNF-α but in low quantities (Martirosyan and Rica, 2011). *Brucella* can hijack the DCs by their effectors to reduce their activation and then modulate their efficiency to produce inflammatory cytokine and to mediate T cells activation (Gorvel et al., 2014). The TNF-α is involved in the macrophage activation and increases their killing activity against *Brucella* (Baldwin et al., 1993). The administration of additional IL-12 increased the immune control of the infection with *Brucella* in mice (Sathiyaseelan et al., 2000). Mice deficient for the IL12p40 subunit of the IL-12 and IL-23 display a reduced control of *Brucella* infection. The loss of control results rapidly in increased damages to the infected tissue (Ko et al., 2002). The secretion of IL-12 in conjunction with TNF-α results in the production of IFNγ (Baldwin and Goenka, 2006) that is a keystone of the immune response against *Brucella*. In response to *Brucella*, the IFNγ can be secreted by both innate immune cells such as $\gamma\delta$ T cells and natural killer cells and adaptive immune cells such as $CD4^+$ and $CD8^+$ T cells (Martirosyan and Rica, 2011). Mice deficient for this cytokine or the interferon regulatory factor 1 are not able to control the infection and die from it (Murphy et al., 2001). These experiments with deficient mice for key factors of the T_H1 show how important these factors of the T_H1 response are crucial in the immunity against *Brucella*.

The adaptive immune response

The $CD4^+$ and $CD8^+$ T lymphocytes are both producers of IFNγ upon intra peritoneal (IP) infection with *Brucella* but their contribution is not equal. The production of the IFNγ has to be mediated by the $CD4^+$ T cells for an efficient control of the infection. The IFNγ producing - $CD8^+$ T lymphocytes are not sufficient to perform this task (Vitry et al., 2012). Moreover, *Brucella* by its BtpB effector can interfere with the cytotoxic activity of the $CD8^+$ T cells (Durward et al., 2012). A subset of the activated $CD4^+$ T cells drives the development of the protective T_H1 immunity. The IFNγ produced activates the killing properties of the effector cells. The infected cells are targeted by the cytotoxic $CD8^+$ T and γδ T cells (Martirosyan and Rica, 2011). These key effectors are essential for *Brucella* clearance and to develop an immune memory against secondary exposure (Martirosyan and Gorvel, 2013). T_H1 response also induce the IgG isotype switch of the B cells. However, B cells have been shown to have a negative impact on the control of *Brucella* (Goenka et al., 2011). The fully efficient memory is only achieved after an exposition of live *Brucella* in an IP infection model. This protection is mediated by the IFNγ producing - $CD4^+$ T lymphocytes and the humoral immunity (Vitry et al., 2014b). In an IP model of infection, the T_H2 and T_H17 seems not to play a significant role in the presence of an effective T_H1 response (Vitry et al., 2012).

The intranasal infection with *Brucella*

To discover key factors involved in the immunity of the host against *Brucella*, most of the in vivo experiments have been performed through IP model of infection. This approach is practical but non physiological. Following IP infection, *Brucella* colonize the organs in a matter of minutes. This model of infection is not relevant of what happens in nature. As a reminder, *Brucella* infections most often occur through the gastrointestinal tract or the respiratory tract that are mucosal sites. In an intranasal (IN) model of infection, the infection is localized in the lungs for the first 36 hours. The infection progressively disseminates to other organs maybe through the lymph nodes (Archambaud et al., 2010). A recent study from our group (Hanot Mambres et al., under submission) described the immune responses induced after an IN infection with *Brucella melitensis*. They reported the involvement of both the T_H17 response and γδ T cells in the control *Brucella* at the early phase of infection. Deficient mice for components implicated in T_H17 responses such as the γδ T cells, the IL-17 receptor and the subunits IL12p40 and IL23p19, were all more susceptible to the infection with *B. melitensis*. However, the humoral immunity has no significant impact on the *Brucella* load in the organs. These results illustrate the importance of choosing the appropriate model of infection in the field of immunity. The immune responses against *Brucella* happening in the lungs at an early stage of infection are still unclear and the effectors still need to be characterized.

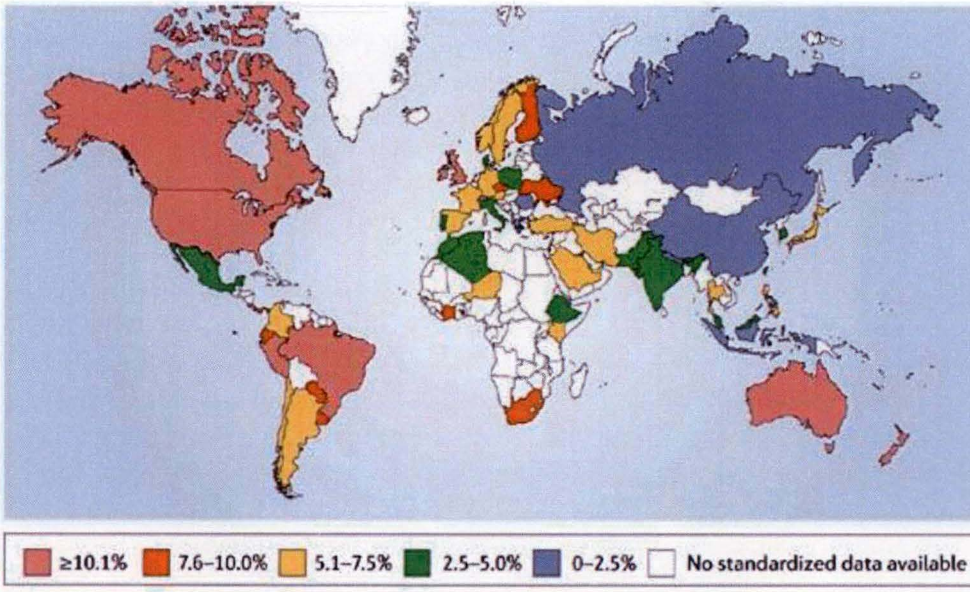


Figure 9: **Global prevalence of asthma in the world.**

The numbers indicate the prevalence of asthma in each country. Asthma is predominant is western countries however data from Africa and Asia are lacking or underestimated.
(Devereux, 2006)

4th Part:

Allergic asthma

Asthma is a chronic inflammatory disorder affecting the conducting airways of the lungs. The principal clinical features of asthmatic patients are a reversible airflow reduction due to structural remodeling of the airways, airway hyper responsiveness (AHR) to non specific stimulation, increased secretion of mucus and smooth muscle constriction. This chronic pathology affects the quality of life of asthmatic patients. The affected people are restricted in their activities (Holgate and Polosa, 2008) and asthma also increases their susceptibility to secondary infections (Habibzay et al., 2013).

The epidemiology

The prevalence of asthma has steadily increased since 1960. Asthma is now a worldwide pathology affecting more than 235 million people (Devereux, 2006) (WHO, 2013) (Figure 9). It is estimated that the asthma prevalence will undergo a 50% increase every 10 years. This disease affects more severely the western countries but also impacts the developing countries (Braman, 2006). Asthma is considered as a heterogeneous pathology that can be divided in different phenotypes. Allergic asthma also called atopic asthma is the most frequent phenotype in humans. This phenotype is associated with a T_H2 immune response against innocuous antigens (Wenzel, 2012). However, the factors and the mechanisms underlying the pathogenesis of allergic asthma is still unclear and a matter of debates (Holgate and Davies, 2009).

The etiology of allergic asthma

Allergic asthma is a complex multifactorial disorder. The pathogenesis of allergic asthma is influenced by both genetic and environmental components. Multiple genes and associated alleles have been linked with the allergic asthma phenotype such as genes implicated in the expression of T_H2 cytokines, microbial recognition and muscle relaxation (Vercelli, 2008).

The allergic asthma pathogenesis occurs most often in childhood. There is increasing evidence that the immune landscape of the lung is shaped early in life or even prenatally. Infection with respiratory syncytial virus (RSV) in childhood have been shown to promote atopy by modulation of the lung immunity (Kusel et al., 2007). The lifestyle in western countries has changed leading to reduced exposure to certain pathogens. The hygiene hypothesis postulates that living in a microbial rich environment confers a relative protection against Th2-mediated allergic diseases by inducing frequent Th1 responses (Strachan, 1989). This change of lifestyle was also associated with a modified diet which in turn with the emergence of antibiotics could lead to a drastic change of our microbiota (Devereux, 2006). The microbiota is an important factor in the maintenance of the homeostasis in the lungs. Changes of the microbiota could have an impact on the physiology of the lungs and then promote asthma (Blaser and Falkow, 2009). These hypotheses could explain the predisposition of some individuals to develop an allergic asthma.

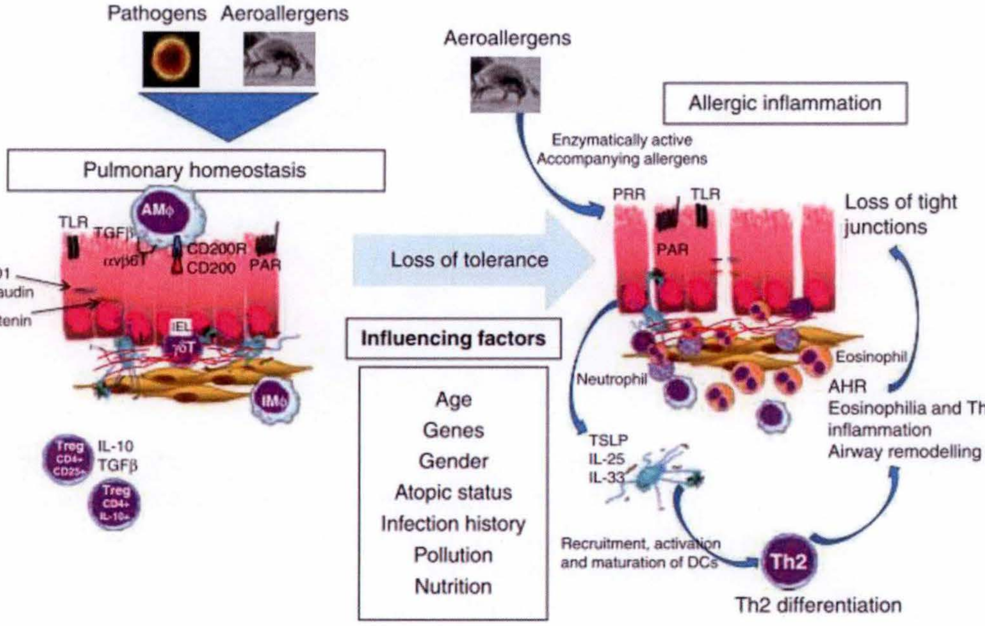


Figure 10: **Mechanisms of sensitization and induction of allergic asthma.**

The steady state condition of the lungs is maintained by the alveolar macrophages, the interstitial macrophages, the γδ T cells and the T reg lymphocytes. The lung homeostasis is the result of tolerance to innocuous environmental antigens and commensal microbiota. Some pathogens and allergens have the capacity to break this tolerance and promote an inflammatory response in the lungs. The pathogenesis of allergic asthma is influenced by both genetic and environmental components. The allergens and the associated damages are recognized and induce a T_H2 immune response. The allergen is encountered again and its triggers the activity of T_H2 effector cells emphasizing the inflammatory state and associated phenotypes.

AM, alveolar macrophages, IM, interstitial macrophages, Treg, regulatory T lymphocytes, γδ T, γδ T lymphocytes, PRR, Pattern Recognition Receptor, TLR, Toll like receptor, T_H, T helper lymphocytes, AHR, Airway hyper responsiveness
(Lloyd and Murdoch, 2010)

House dust mites

In susceptible individuals, some allergens such as the animal danders, the pollen, molds like *Alternaria alternata*, and house dust mites (HDM) have the capacity to dysrupt the the lung homeostasis promoting the airway inflammation. HDM like *Dermatophagoides pteronissinus* or *Dermatophagoides farinae* are potent inducers of the allergic asthma phenotype. These aeroallergens are able to breach the mucosal barrier of the lung by their protease activity. This proteolytic property induces injuries of the epithelium and generates associated DAMPs (Lloyd and Murdoch, 2010) (Figure 10). The functionnal homology of the protein Derp2 with the adaptor MD2 of the TLR4, increases its activity to recognize the LPS (Trompette et al., 2009). The HDM also contains allergens potentially recognized by the TLRs. The HDM total extracts are generally contaminated with TLR agonist such as LPS (Lloyd and Murdoch, 2010). Moreover, the HDM possess B-glucan motifs which are known signals to induce the chemotaxis of the DCs to the lungs (Nathan et al., 2009). These DCs increase the pool of DCs in the lungs increasing the likelyhood of the exposure of DCs to the allergenic environment generated by the HDM (Lambrecht and Hammad, 2009).

Allergic asthma: the immune response

During the sensitization step, the aeroallergens dysrupt the homeostasis of the lung notably if they possess protease activity. This kind of activity generates danger signals that are recognized. In response the epithelium releases cytokines and chemokines like IL-33 and the thymic stromal lymphoprotein (TSLP). These will stimulates mast cells, basophils activity but also induce the infiltration of eosinophils and later the differenciation of the T_H2 $CD4^+$ T cells (Lambrecht and Hammad, 2009).

The mast cells are important effector cells in allergic asthma. Their activation triggers the synthesis of its inflammatory effectors contained in granules. These granules are preformed with a wide range of inflammatory compounds like biogenic amines (histamine, serotonine, dopamine,...), proteases (cathepsins, trypases, chymases, granzyme B,...) and T_H2 cytokines (IL-5, IL-6 and IL-13) (Wernersson and Pejler, 2014).

The basophils are thought to be an important inducer of the T_H2 as they are early producers of IL-4 and can even act as APC. Through their secretion of IL-4, they can have a major role in the cell polarization notably in protease induced allergic asthma (Lambrecht and Hammad, 2009).

The early infiltration of eosinophils and neutrophils is regulated by the IL-17/IL-23 axis of the T_H17. The T_H17 response enhances the T_H2 cells capacity to recruit granulocytes like neutrophils and eosinophils (Wakashin et al., 2008). Both of these cells can form extracellular traps against pathogens. The eosinophils granules contain cationic proteins and peroxidase that once released will clear the inflammatory stimuli. These cells are associated with airway damages and overall inflammation (Felton et al., 2014).

The inflammatory context together with the TLR recognition and associated downstream signalling promote the activation of the DCs to trigger a T_H2 response (Lambrecht and Hammad, 2009). The activated DCs migrate to the mediastinal draining lymph nodes of the lungs and drive the polarization of the naive $CD4^+$ T cells into $CD4^+$ T_H2 cells. By the homing process, these mature T_H2 cells come back to the inflammed tissue to organize the T_H2 response. The T_H2 cells migrate also to the B cells germinal center to favor IgE isotype switch of specific B cells. The activation of the B cells induces their proliferation and directs the IgE isotype switch. The activated B cells secrete specific IgE of high affinity. These IgE will link with the FcεR of the mast cells and basophils. People affected by allergic asthma will feature high amounts of both total and specific IgE at the inflammation site and in the blood (Wu and Zarrin, 2014).

Secondary exposures to the same allergens induces a rapid and emphasized T_H2 response. The allergen cross links with the IgE at the surface of both mast cells and B cells. The mast cells degranulate and liberate their inflammatory effectors and trigger the synthesis of leukotriene, prostaglandine and vasoactif mediator. As a results, the inflammation is autoamplified by the substances, chemokines and cytokines released by the T_H2 effector cells. This inflammation upon chronic challenges of the allergen lead to the clinical trait of allergic asthma with airway obstruction by mucus hypersecretion and by bronchoconstriction, vasodilation and eosinophilic infiltration associated with AHR (Holgate, 2012).

OBJECTIVES

Brucella melitensis is a facultative intracellular bacterium *causing* a worldwide zoonosis called brucellosis. One of the potential routes of entry for *Brucella* is through the respiratory tract after inhalation of aerosols. Interestingly, the course of infection of *Brucella* in the lung appear only transient (Atluri et al., 2011). *Brucella* does not persist at high levels in the lungs as other causative agents of ganulomatous disease such as *Mycobacterium tuberculosis,* the agent of tuberculosis (Ernst, 2012). A previous study (Hanot Mambres et al., in preparation) suggests that T_H1 and T_H17 mediated immune response are implicated in the control of *Brucella* in the lungs. In order to define the parameters affecting this protective immune control, we used a cross-pathology model implicating house dust mites (HDM)-induced allergic asthma as chronic bystander pathology. Successive exposures to HDM extracts is considered as relevant of an allergic asthma phenotype in humans (Wenzel, 2012) (Marichal et al., 2010). The asthma pathology result from the development of a Th2 immune response. This latter is well-known to antagonize T_H1 anti bacterial immune response in several models.

We will characterize the impact of asthma on the mouse ability to control the infection by *B. melitensis.* To do so, we will evaluate the bacterial load of the spleen, liver and lungs by measuring CFU at different timepoints. To decipher the mechanisms implicated in this expected impact, we will use mice deficient for key components of the immune responses. Finally to monitor the immune responses and compare them between the different conditions, we will perform:

- The measurement of the Ig titers through ELISA.
- The characterization of the infected cells and the recruitment of cells in the lungs by immunohistofluorescence.
- The analysis and characterization of the immune cells populations and cytokines produced in the lungs by flow cytometry.

Asthmatic people are also an important target population for vaccination as they are generally more susceptible to infection by a wide range of pathogens and so need enhanced immunity (Edwards et al., 2012). However, it has been showed that T_H2 immune responses induced by helminths can have a negative impact on vaccination (Apiwattanakul et al., 2014; Elias et al., 2006a). So we want to evaluate if allergic asthma through its T_H2 immune response could have an impact on the development of protective immune memory following intranasal *Brucella* infection.

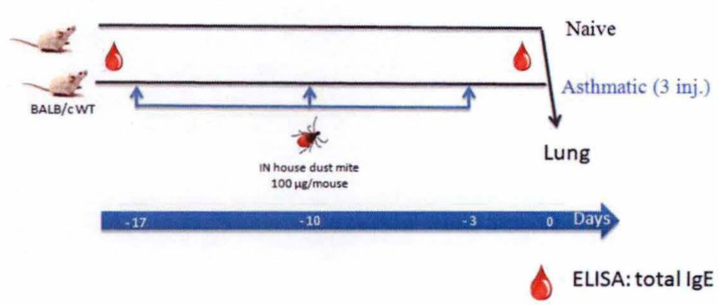
RESULTS:

I. DEVELOPMENT OF ASTHMA MODEL

In this study, we used an House Dust Mite (HDM) model to induce a T_H2 allergic asthma phenotype in BALB/c mice. This model was established by the Lambrecht research group (Hammad et al., 2009) and used in previous studies to analyze the immunological responses in allergic asthma (Marichal et al., 2010). The induction protocol consists in repeated intranasal injections of 100µg/50µl of total HDM extracts of *Dermatophagoides farinae*. The injections are done once a week for 3 weeks. The principal features of allergic asthma induced by this model have already been described by the research group of Fabrice Bureau (Marichal et al., 2010)[2].

Serological analysis:

The measurement of the IgE levels in the serum of the mice, allows us to quantify the potency of the HDM extract to induce a T_H2 immunity. The total IgE titers in the peripheral blood of the mice were measured before and after the last HDM injections, at the described peak of the T_H2 immune response (Marichal et al., 2010). As expected, the total IgE titers in serum were significantly increased after the HDM treatment. This result suggests that the repeated HDM exposures induced well a T_H2 immune response (Figure 1).



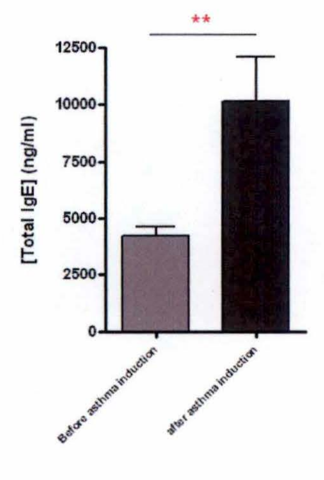
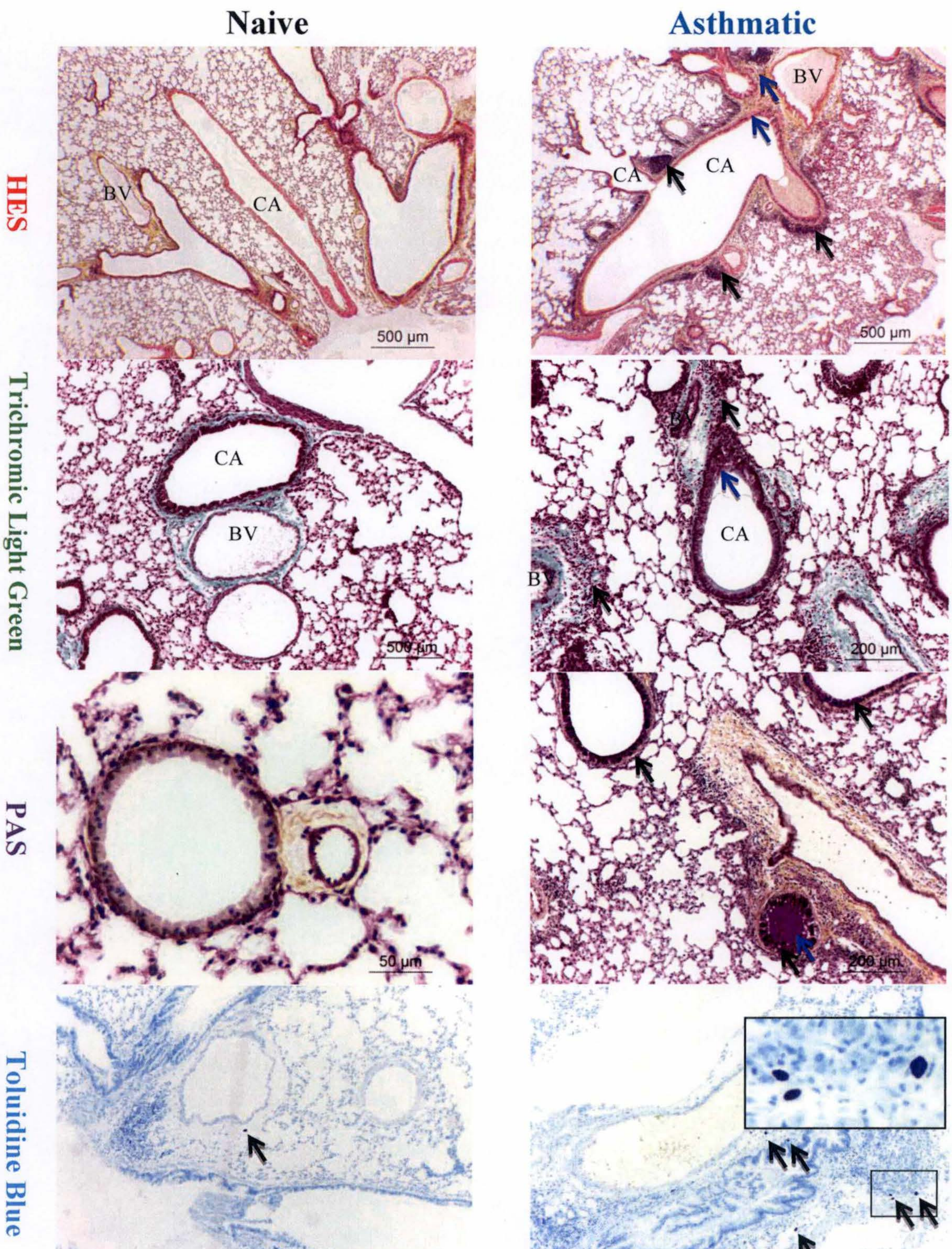


Figure 1. Evaluation of the serum total IgE titers. Peripheral blood was taken just before and 3 days after the induction of asthma by 3 consecutive intranasal injections of 100µg of HDM/ mouse on day -17, -10 and -3. Measurements of total IgE were performed by ELISA tests on serum samples of each individual mouse (N=3). The bars are the mean ± SD. Significant differences are denoted by asterisks (**$p < 0.01$).

[2] We actively collaborate with this group but we first wanted to confirm these data in our laboratory conditions. In order to do so, we controlled some of the key features of the allergic asthma such as the IgE titers, the remodeling and the activation of the airway epithelium and the recruitment of some immune effector cells.



Naive
Asthmatic
HES
Trichromic Light Green
PAS
Toluidine Blue
BV
CA
500 µm
200 µm
50 µm

Histological analysis:

The structural modifications of the airways and the recruitment of immune cells were evaluated by classic histology and immunohistofluorescence analysis of several lungs sections per mouse (n=6). Therefore, the lungs were compared between control BALB/c wild type (WT) mice receiving 3 PBS injections and asthmatic BALB/c WT mice receiving 3 HDM injections (see Figure 1). Using Hematein-Eosin-Safran (HES) and Trichromic Light Green stainings, we observed (in Figure 2) massive infiltration of cells notably eosinophils at the periphery of the conducting airways (CA) and blood vessels (BV). We also observed an epithelial thickening of the conducting airways only in the asthmatic condition. The inflammation of the lung airways seems not to affect the alveoli in none condition. As the mucus is composed of saccharides and glycoproteins, we detected its presence with a Periodic Acid Schiff (PAS) staining. The goblet cells were activated and hyperplasic with an enhanced production of mucus leading to the obstruction of some of the bronchiols of the asthmatic mice. The metachromasic staining (Toluidine Blue) of the mast cells granules highlighted their recruitment and degranulation under asthmatic condition (Figure 2).

The immunohistofluorescence analysis showed massive recruitment of cells (identified using DAPI staining) at the proximity of the conducting airways and blood vessels. An important part of them were granulocytes (expressing Ly-6G marker) that can be both neutrophils and/or eosinophils (Figure 3). These data are similar to those published previously and are relevant of an allergic asthma phenotype induced by an HDM treatment.

Figure 2. Histological analysis of lung tissue after allergic asthma induction.
Both lungs of naive and asthmatic mice were collected on day 0 and examined by histology. Lungs sections were stained by HES, Trichromic Light Green, PAS - Hemalun – Safran and Toluidine Blue. Lung sections from naive and asthmatic mice are in the right and left column respectively. In HES and Trichromic Light Green stainings, some cells infiltrations and epithelium thickenings are pointed by black and blue arrows respectively. In PAS stainings, black arrows point goblet cells hyperactivation and blue arrows point secreted mucus. In Toluidine Blue, Mast cells are pointed by black arrows. The region selected is a 4 fold enlargement. Each image chosen is representative. (BV = Blood Vessel, CA = Conducting Airway)

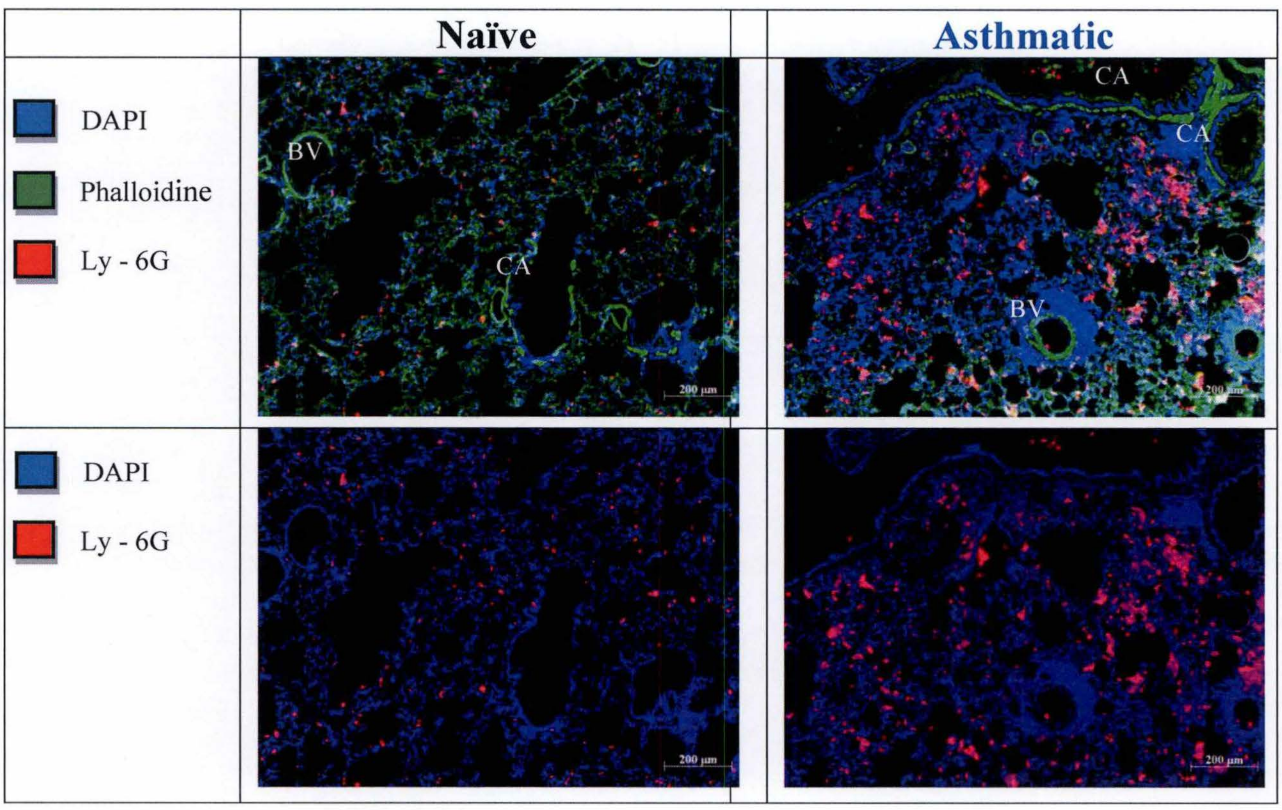


Figure 3. Immunohistofluorescence analysis of lung tissue after allergic asthma induction. Analysis of DAPI (DNA), Ly-6G expressing cells and phalloidin (actin) expression in the lungs. BALB/c WT asthmatic mice were compared with naive mice as previously described. The lungs were collected on day 0 and examined by immunohistofluorescence. The lung sections from naive and asthmatic mice are in the right and left column respectively. Left column describes the markers used. Scale bars are 200µm. Each image chosen is representative. (BV = Blood Vessel, CA = Conducting Airway)

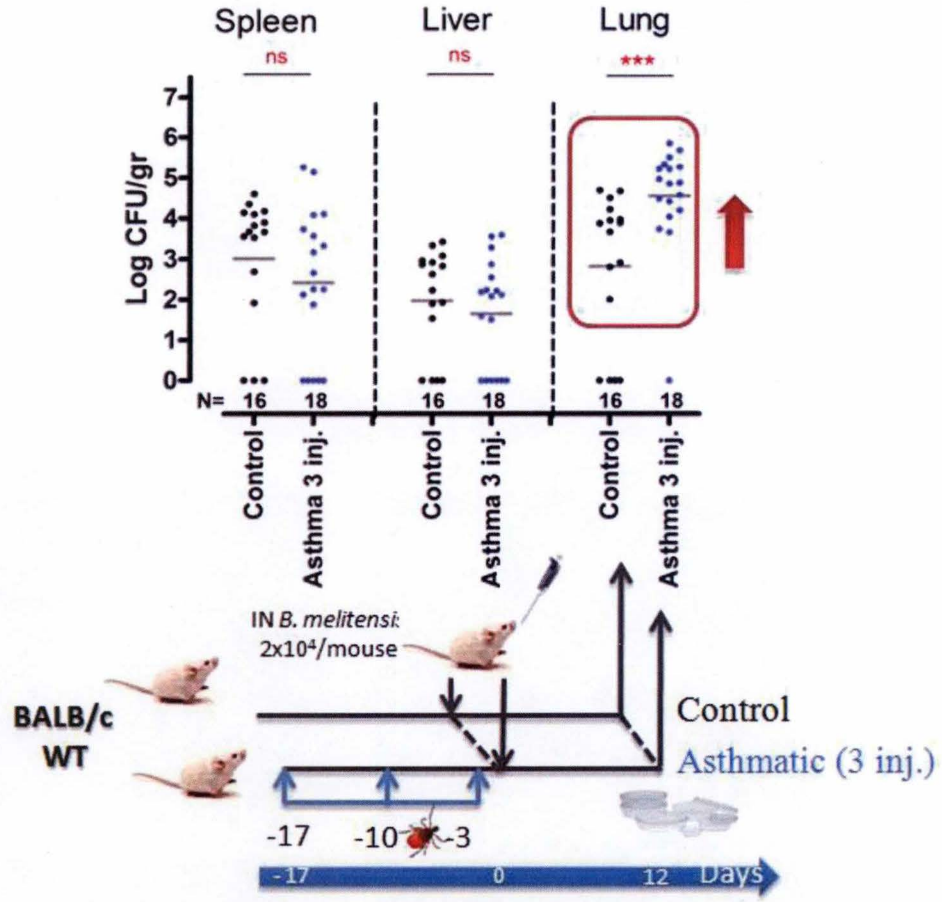


Figure 4: **Local impact of asthma on the *Brucella* burden in the lungs**. CFU counts analysis, the data represent the number of CFU per gram of organ. Horizontal gray bars are the mean. These results are representative of four independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

II. IMPACT OF ALLERGIC ASTHMA ON THE COURSE OF *BRUCELLA MELITENSIS* INFECTION IN MICE

Allergic asthma reduces locally the ability of the BALB/c wild type mice to control *Brucella melitensis* infection in the lungs.

The cross-pathology model was first performed on 2 groups of BALB/c WT mice : control mice treated with PBS and asthmatic group treated with HDM. Both groups were infected intranasally with $2x10^4$ CFU of *B. melitensis*. The *Brucella* burden was evaluated by counting CFU in the spleen, the liver and the lung of the mice at 12 days post infection. This timepoint was selected to monitor if a time shift occurs or not in the timing of *Brucella* dissemination from the lungs to the spleen and liver. The *Brucella* load in the spleen and the liver appears similar in both groups. Interestingly, the CFU count in the lungs of asthmatic mice was significantly (***) higher than in control group (Figure 4), suggesting that the preexisting T_H2 environment favor Brucella growth and/or has a negative impact on the immune control of *Brucella*. This phenomenon seems restricted to the lung compartment.

HDM challenge during the course of infection increase the positive impact on *Brucella* growth in lung

We next wanted to optimize the protocol of HDM treatment to increase the impact of asthma in further experiments. Therefore, we increased the number of HDM challenges before the infection, increasing maybe the preexisting allergic asthma phenotype of the mice. We compared the 3 injections HDM treatment with a 6 injections HDM treatment. The two groups were infected and evaluated at the same time. We observed no significant differences between the two models for the three organs tested (Figure 5A).

We then assessed if we could modify the impact of asthma by pursuing the HDM treatment during the course of infection of *B. melitensis*. We add only one HDM challenge during the course of infection to respect the one-week rest time and to keep the evaluation timepoint for comparison. The additional HDM injection significantly increased (*) the *Brucella* load in the lungs (Figure 5B). The allergic asthma decreases the ability of the mouse to control the infection but this impact remains restricted to the lung so far. As the model with 3 injections before and repeated injections during the course of infection, displayed an higher impact, we will keep it for further experiments.

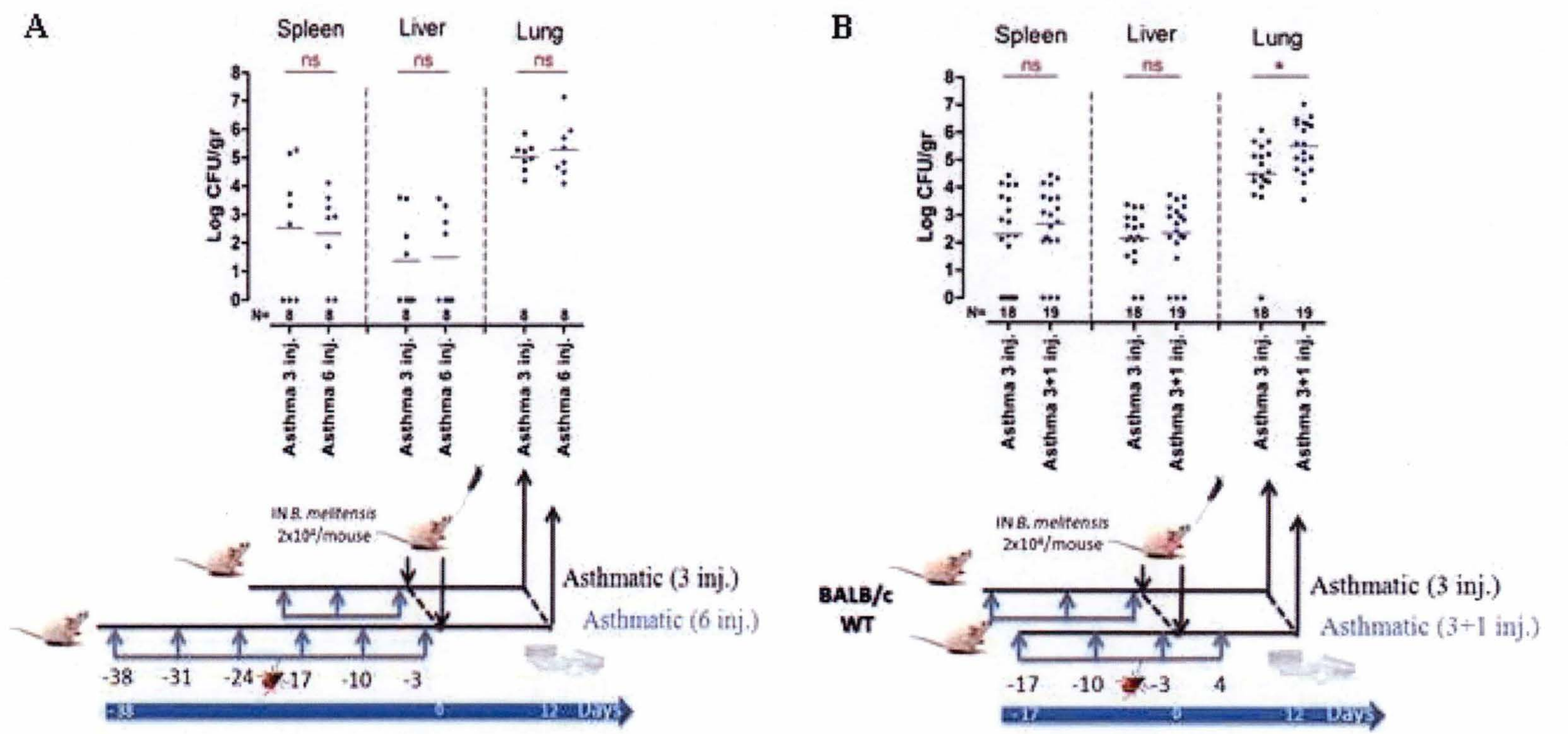


Figure 5: **Modifications in the HDM treatment protocol to enhance its impact**

CFU counts analysis, the data represent the number of CFU per gram of organ. Comparison between two models of HDM treatment. A. Comparison between 3 and 6 HDM injections before the infection. B. Comparison between 3 and 3 HDM injection before + 1 injection after the infection. Horizontal gray bars are the mean. These results are representative of at least two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

A

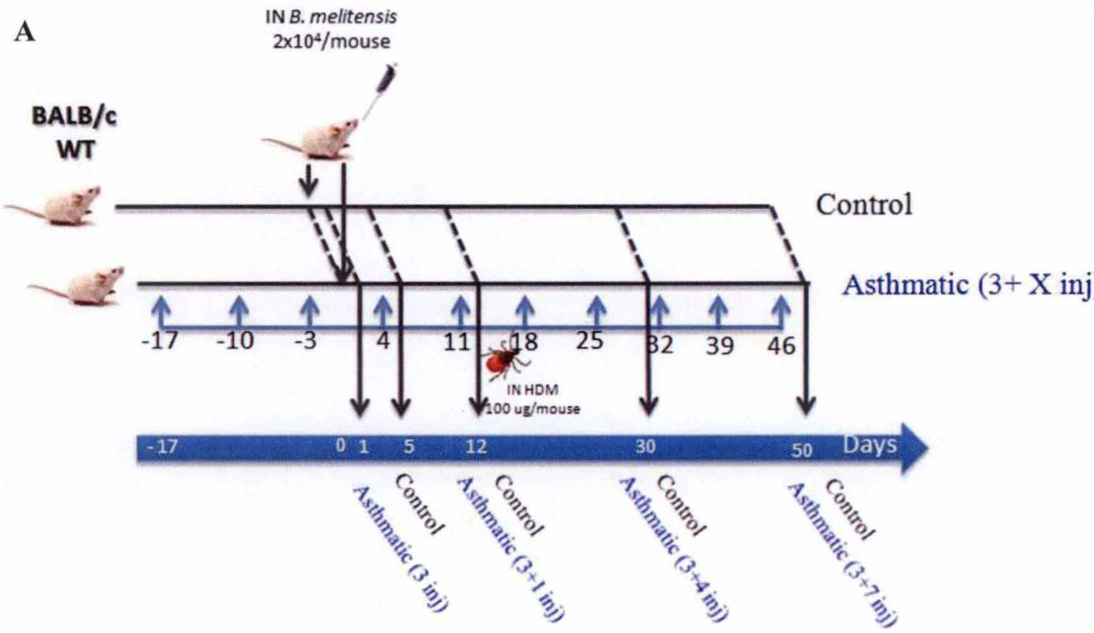


Figure 6: **Course of infection of *Brucella melitensis* in BALB/c WT mice (Part 1)**
A. Scheme representing the steps and the conditions of the experimentation B. CFU counts analysis and associated curve. The data represent the number of CFU per gram of organ. The *Brucella* load was evaluated in the spleen, the liver and lung at 1, 5, 12, 30 and 50 days post infection. Horizontal gray bars are the mean. The bars are the mean ± SD. These results are representative of two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

Chronic HDM challenges favor *Brucella* persistence in lung

Previous result demonstrate that HDM treatment-induced allergic asthma decreases the control of the mouse to the infection by *Brucella melitensis*. To describe more deeply this impact, we characterized the course of infection from early timepoints to the chronic phase of the infection in both conditions. The mice were infected by an intranasal injection of $2x10^4$ CFU of *B. melitensis*. After intranasal infection, the *Brucella* load was evaluated in the spleen, liver and the lungs at 1, 5, 12, 30 and 50 days post infection (Figure 6). The asthmatic mice were challenged by an HDM injection every week all along the course of infection.

For the first 24 hours, the *Brucella* load in the lung remained the same as the infectious dose ($2x10^4$ CFU). The control mice only infected started to control the infection from 5 days post infection. The *Brucella* load dropped constantly at the point to be undetectable (detection threshold = 10 CFU) at 30 days. In contrast, the asthmatic mice showed an increased *Brucella* burden in the lungs from 5 to 30 days post infection with a peak at 12 days.

The dissemination from the lungs to the spleen and the liver is slow and progressive. It starts to be detectable in some mice at 5 days and reaches a maximum at 12 days post infection. Interestingly, there was a transient increased in the *Brucella* burden in the spleen of asthmatic mice at 30 days compared with the control group. However, except this point, there is no difference in the *Brucella* load of the spleen and the liver from both groups of mice whatever the time considered (Figure 6). All together these data shows that the allergic asthma impairs locally the ability of the mouse to control *Brucella* already at early time post infection. The asthmatic condition also increases the capacity of *Brucella* to persist in the lungs for a longer time.

Allergic asthma also impact *B. melitensis* infection in C57BL/6 WT resistant mice

The choice of the mouse strain has a great importance in both the *Brucella* model and the HDM-induced allergic asthma model. Some strains have a bias in the T_H1-T_H2 balance due to their different genetic background. The BALB/c mice are a reference strain to study T_H2 mediated inflammatory disorders (Bates et al., 2009). They are more relevant of an atopic individual with their T_H2 bias (Paul and Zhu, 2012). BALB/c mice are also more susceptible to *Brucella* infection (Vitry et al., 2014b). In contrast, C57BL/6 mice are well-described to be resistant to *Brucella* infection (Vitry et al., 2014b), displaying a TH1 bias and so are more resistant against the development of T_H2 mediated immune responses (Paul and Zhu, 2012). In an attempt to generalize our results, we have transposed the cross-pathology model to C57BL/6 WT mice.

As expected, the impact of HDM treatment on *Brucella* infection is reduced in a C57BL/6 WT background as compared to BALB/c WT mice (Figure 7). This is correlated with reduced total IgE levels in the serum of these mice after a 3+1 HDM treatment (Figure 8). The impact of the allergic asthma is transposable to C57BL/6 WT mice but need an additional HDM challenge during the course of infection. The 3+1 HDM treatment is the most potent model to show differences in these mice (Figure 7).

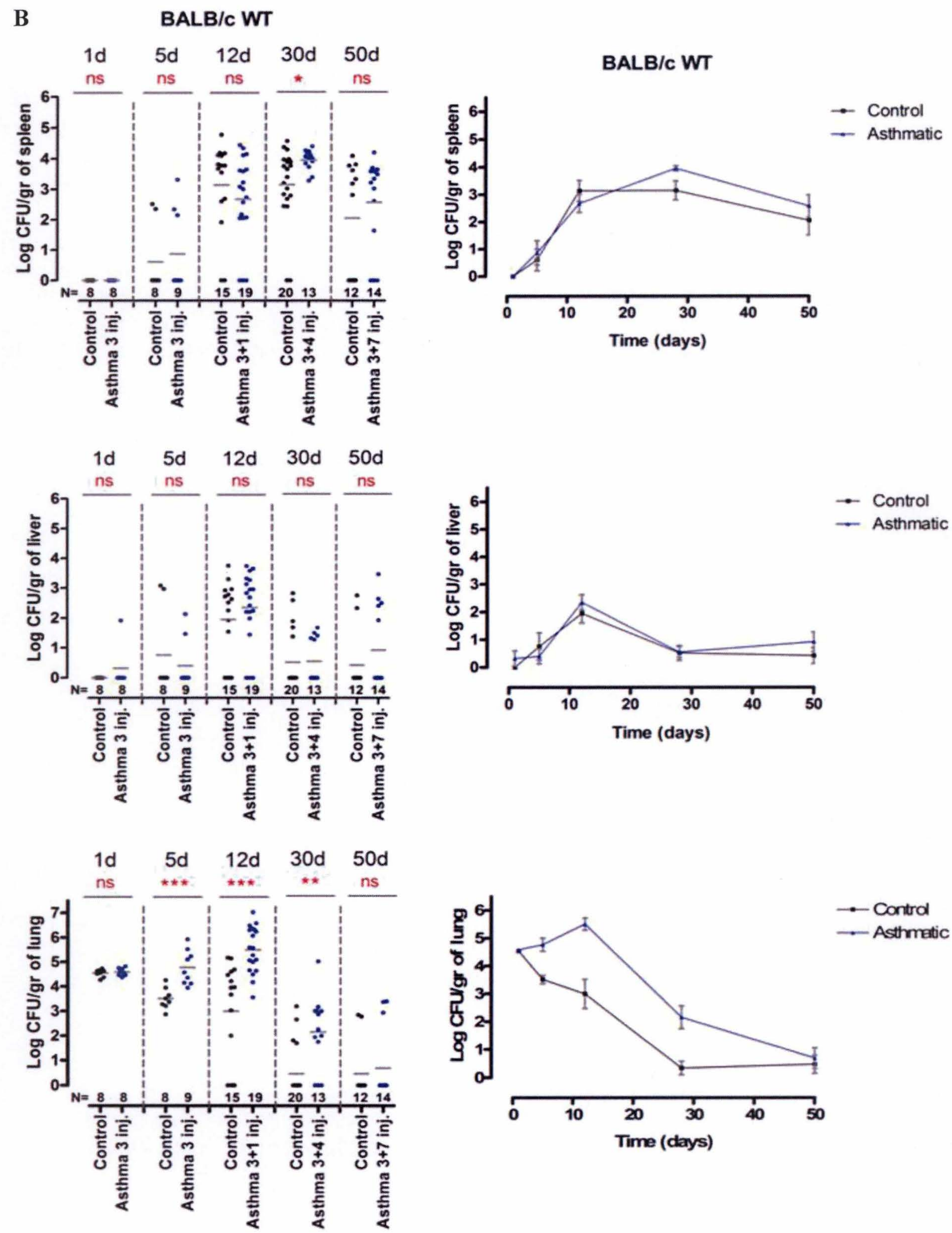


Figure 6: **Course of infection of *Brucella melitensis* in BALB/c WT mice (Part 2)**

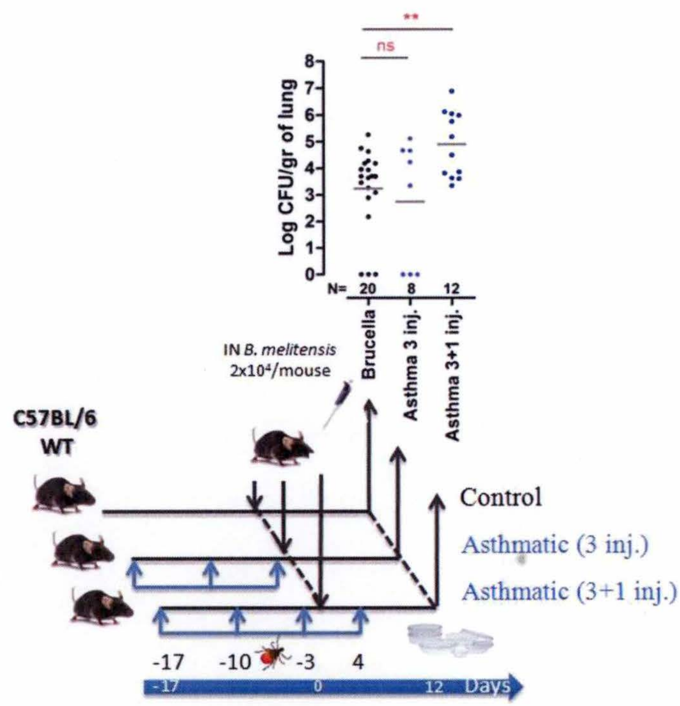


Figure 7: **The impact of asthma on *B. melitensis* in C57BL/6 WT mice**

CFU counts analysis at 12 days post infection, the data represent the number of CFU per gram of lungs. Comparison between 3 HDM injections before and 3 + 1 injections after the infection for the asthmatic groups. Horizontal gray bars are the mean. These results are representative of at least two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

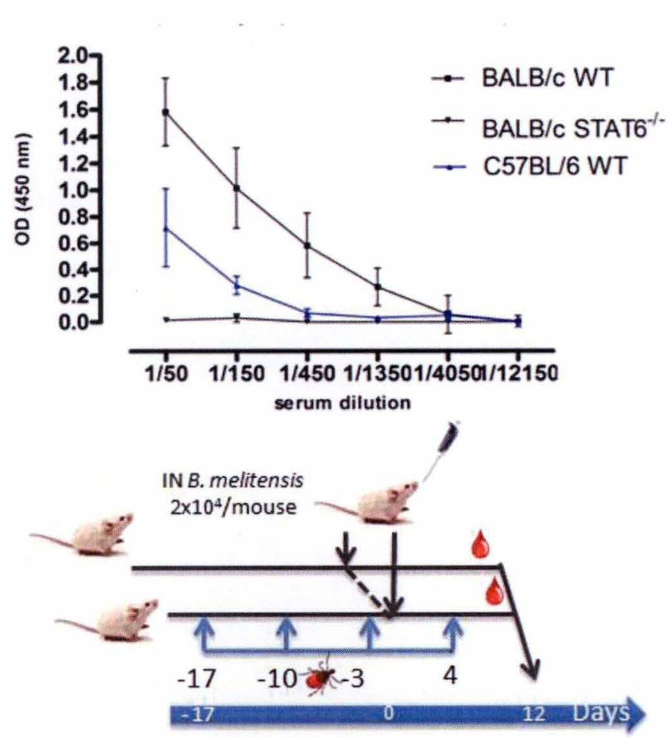


Figure 8: **Measurements of the total IgE in BALB/c WT, STAT6$^{-/-}$ and C57BL/6 WT mice**

Peripheral blood was taken 12 days post infection in BALB/c WT, STAT6$^{-/-}$ and C57BL/6 WT asthmatic mice. Data represent the OD measurements at 450nm per serum dilution. Bars are the mean ± SD. These results are representative of two independent experiments.

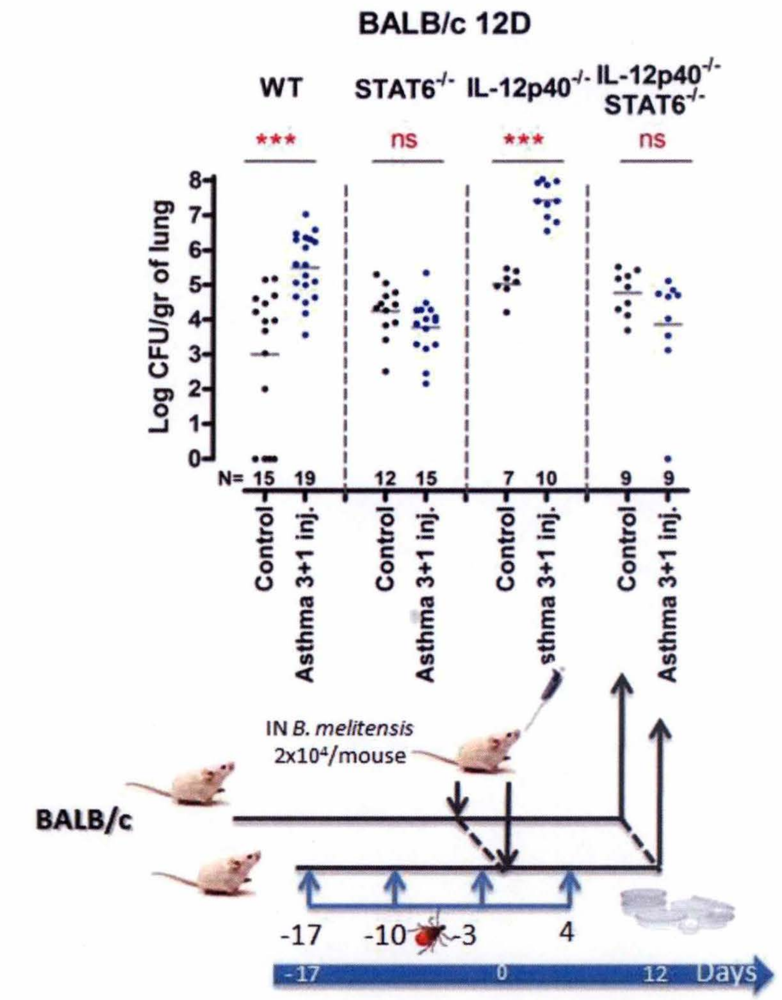


Figure 9: **Comparison between the *Brucella melitensis* infection of BALB/c WT, STAT6$^{-/-}$, IL-12p40$^{-/-}$ and IL-12p40$^{-/-}$ STAT6$^{-/-}$ mice.**

CFU counts analysis, the data represent the number of CFU per gram of organ. Horizontal gray bars are the mean. These results are representative of two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

III. HOW ALLERGIC ASTHMA AFFECTS THE CONTROL OF BRUCELLA INFECTION IN LUNG?

Hypothesis

Based on these results, the two most reasonable hypotheses (not exclusive) to explain the lack of control of *Brucella* growth in the lungs following HDM treatment are:

- The preexisting T_H2 immune response induced by the allergic asthma inhibits the induction of the T_H1 immune response, the main response to control *Brucella*.

- The T_H2 mediated inflammation of the allergic asthma alters the number and/ or the status of the reservoir cells in the lung dampening their efficiency to respond to the infection and to kill the bacteria.

Depletion of T_H1, T_H2 and T_H17 immune responses by the use of deficient mice

To investigate the first hypothesis, we compared the susceptibility of BALB/c mice genetically deficient for the IL-12p40 and STAT6. As a reminder, the transcriptional activator STAT6 is regulated by the IL-4 and is essential for the recruitment of T_H2 effectors and the amplification of the T_H2 response (Paul and Zhu, 2012). The IL-12p40 is a subunit of both the IL-12 and IL-23. Mice deficient for this gene have a partial depletion of both T_H1 and T_H17 immune responses (Williams, 2012). These asthmatic mice were evaluated through the protocol 3+1 HDM injections and the *Brucella* load was evaluated in the lungs 12 days post infection.

STAT6$^{-/-}$ BALB/c mice display similar *Brucella* burden in both control and asthmatic conditions (Figure 9). The loss of impact in STAT6 deficient mice was confirmed at 12, 30 and 50 days post infection (Figure 10). This loss is also correlated with undetectable total IgE level in these mice for the same dilution used for BALB/c WT mice (Figure 8). This result clearly demonstrates that the impact of HDM treatment on *Brucella* is dependent of the development of HDM-induced T_H2 response.

As expected, control BALB/c mice deficient for IL-12p40 have a significant (**) increased *Brucella* burden in comparison to their WT counterparts. Surprisingly, the *Brucella* burden in the asthmatic IL-12p40$^{-/-}$ mice appears higher (***) than in the control condition. Despite the T_H1 and T_H17 depletion context, the impact of asthma is conserved. As previously, the STAT6 deficiency in a IL-12p40$^{-/-}$ context is sufficient to suppress the impact and restore the same *Brucella* load as in both IL-12p40$^{-/-}$ STAT6$^{-/-}$ and IL-12p40$^{-/-}$ control conditions.

These data suggests that the impact of allergic asthma is dependent on the STAT6 signaling pathway but surprisingly, does not rely on the inhibition of the T_H1 and T_H17 by the T_H2 environment.

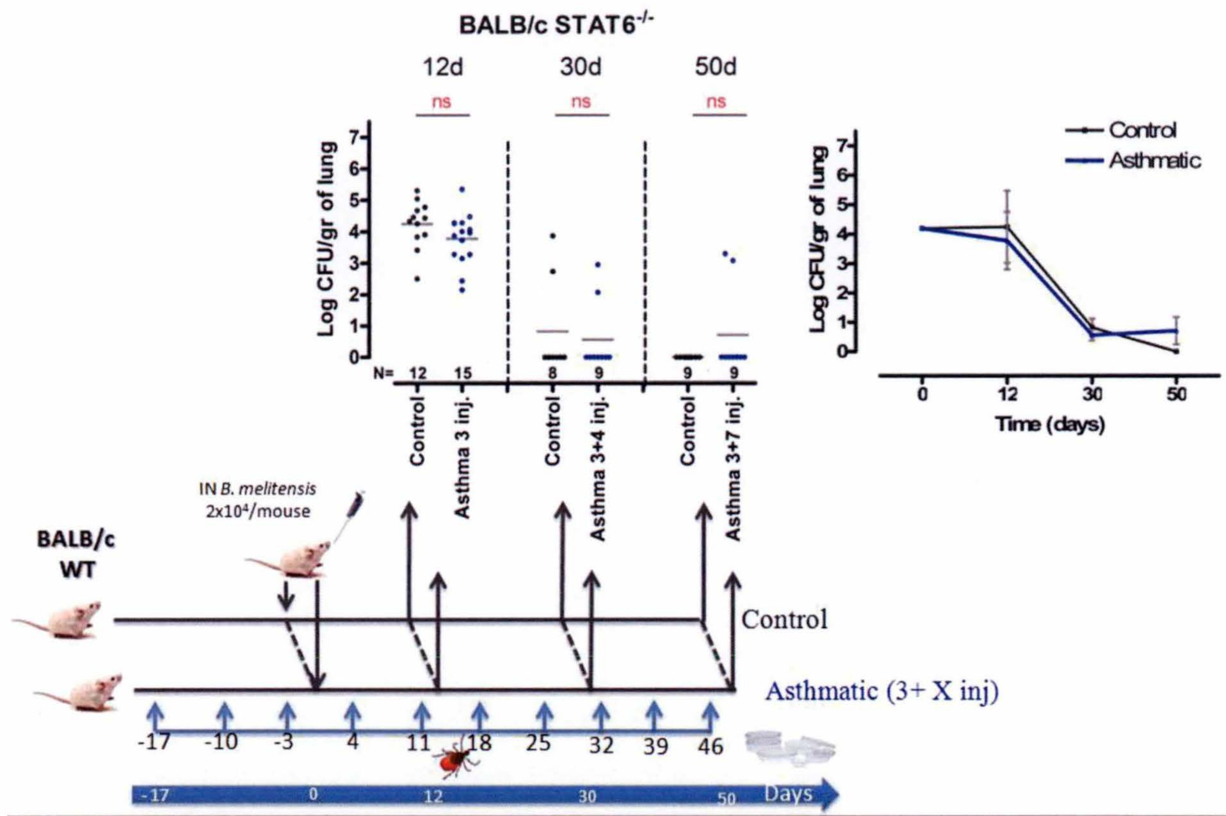


Figure 10: **Asthma impact on *Brucella* lung infection is abolished in BALB/c STAT6$^{-/-}$ mice.** CFU counts analysis and associated curve. The data represent the number of CFU per gram of lung. The *Brucella* load was evaluated in the lung at 12, 30 and 50 days post infection. Horizontal gray bars are the mean. The bars are the mean. These results are representative of two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

To confirm by another approach that allergic asthma does not impair the T_H1 and the T_H17 immune responses, we also compared the percentage of cells expressing key cytokines for T_H1 and T_H17 responses through flow cytometry analyses. Unfortunately, low dose infections with *B. melitensis* in the lungs do not elicit an immune a level of cytokine detectable by flow cytometry. Moreover, the visualization of *Brucella* mCherry fluorescence in the tissue is difficult at low dose infections (detection threshold = 6 Log of CFU/gr of lung) (Copin et al., 2012). High *Brucella* load in the lungs are required to be able to observe *Brucella melitensis* mCherry by histofluorescence. For these reasons, we had to switch from low dose ($2x10^4$ CFU of *B. melitensis*) infection to high dose ($2x10^7$ CFU) infection protocol.

The course of *Brucella* infection following high dose intranasal infection

We therefore assessed if the impact of allergic asthma is reproducible in a high dose infection model. The *Brucella* load was evaluated in the lung of mice at different timepoints during the early course of infection (3, 5 and 12 days post infection) (Figure 11). The *Brucella* burden in the lungs is similar at 3 days post infection. As previously, asthma increases the *Brucella* burden at 5 days post infection. This increase results in up to 8.8 Log of CFU in the lungs of some asthmatic mice. At high infectious dose, the impact is maximal at earlier time point (5 days post infection) and is more transient than in a low dose infection model. Thus, the high dose infection allows the visualization of *Brucella* in the lung tissues but shortens the kinetic of the impact of asthma on the infection in the lungs. This feature will be taken into account for further experiments.

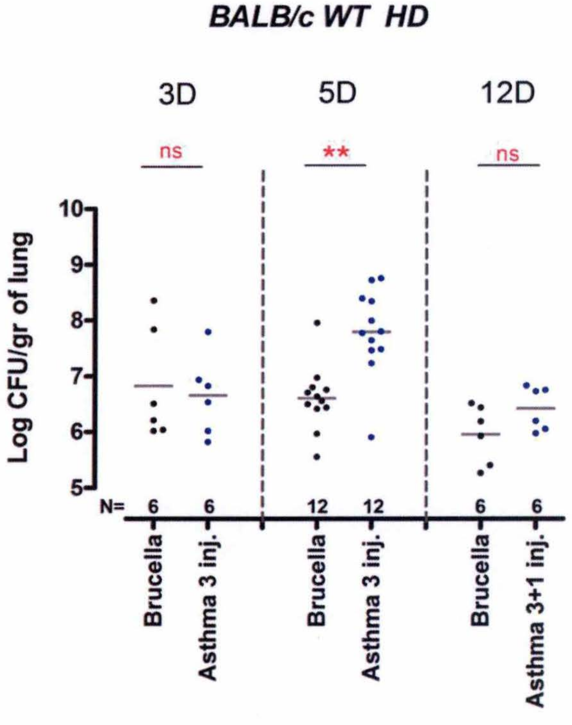


Figure 11: ***Brucella* course of infection with a high dose inoculum**
CFU counts analysis, the data represent the number of CFU per gram of lung. The *Brucella* load was evaluated in the lung at 3, 5 and 12 days post infection. Horizontal gray bars are the mean. These results are representative of two independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

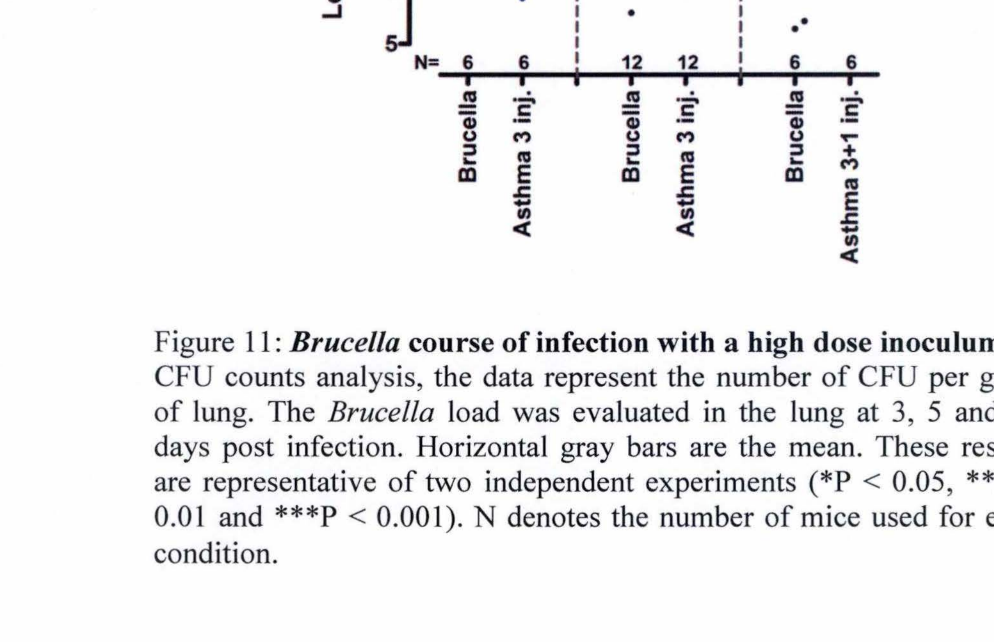


Figure 12: **Frequency of cell producing IFNγ, iNOS or IL-17 in BALB/c WT mice (Part 1)**

Representative gate used and data plots for the IFNγ $^+$ cells population shown in Figure 12 part 2. FSC, Forward Scatter

The production of T_H1 and T_H17 cytokines is not impaired despite the T_H2 context

Using the high dose infection model, we characterized the immune responses induced in the lungs by flow cytometry analysis[3]. We measured the frequency of cells expressing key cytokines for the T_H1 and T_H17 immune responses and characterized their phenotype. We evaluated at 1, 5 and 12 days post infection, the expression of IFNγ, inducible Nitric Oxide Synthase (iNOS) and IL-17 by the immune cells of the lung. Their expression is the result of a natural activation *in vivo* and not the result of stimulation *in vitro*.

The expression of these cytokines and enzyme starts to rise in the infected conditions in comparison with the control only from 5 days post infection (Day 1, data not shown). The production of IFNγ, iNOS and IL-17 is not reduced by HDM treatment. The number of cells expressing iNOS and IL-17 is even increased in the asthmatic infected mice (Figure 12). Moreover, the number of IL-17$^+$ cells in the asthmatic infected mice is too high to be explained by the simple addition of both asthmatic and *Brucella* infected conditions. One experiment also showed the same results with the IFNγ$^+$ positive cells (Data not shown). The important number of IFNγ$^+$ cells in infected mice at 5 days, disappeared at 12 days post infection. A delayed rise in the expression of iNOS in these infected mice followed at 12 days post infection (Figure 12). All together, these data strongly suggest that the T_H2 environment induced by asthma does not impair the mouse to mount a T_H1 or a T_H17 immune response in the lungs in response to *Brucella* infection.

The analysis of some cell surface marker, enables us to some extend to phenotype the cells producing these cytokines and enzyme (Data not shown). The phenotype of these cells is similar whatever the infected conditions. The IL-17 producers are not lymphoid cells as they are negative for both γδ (expressed by γδ T cells) and CD90 (expressed by T cells and NK cells) marker. The IL-17$^+$ cells also express the CD11b$^+$ and Ly-6G^{high} marker at high level, a profile that could match with neutrophils. The nature of the IFNγ producer cells is less clear. The producers of IFNγ are not T cells as they are CD3$^-$ CD4$^-$ TCRβ$^-$. The iNOS producing cells (CD11b^{high} CD11c^{high}) are probably inflammatory DCs. A lot of plausible candidates for the origin of IFNγ have been discarded but the precise phenotype of the producing cells is still under research.

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[3] The flow cytometry protocol and the analysis presets were adapted for the lung.

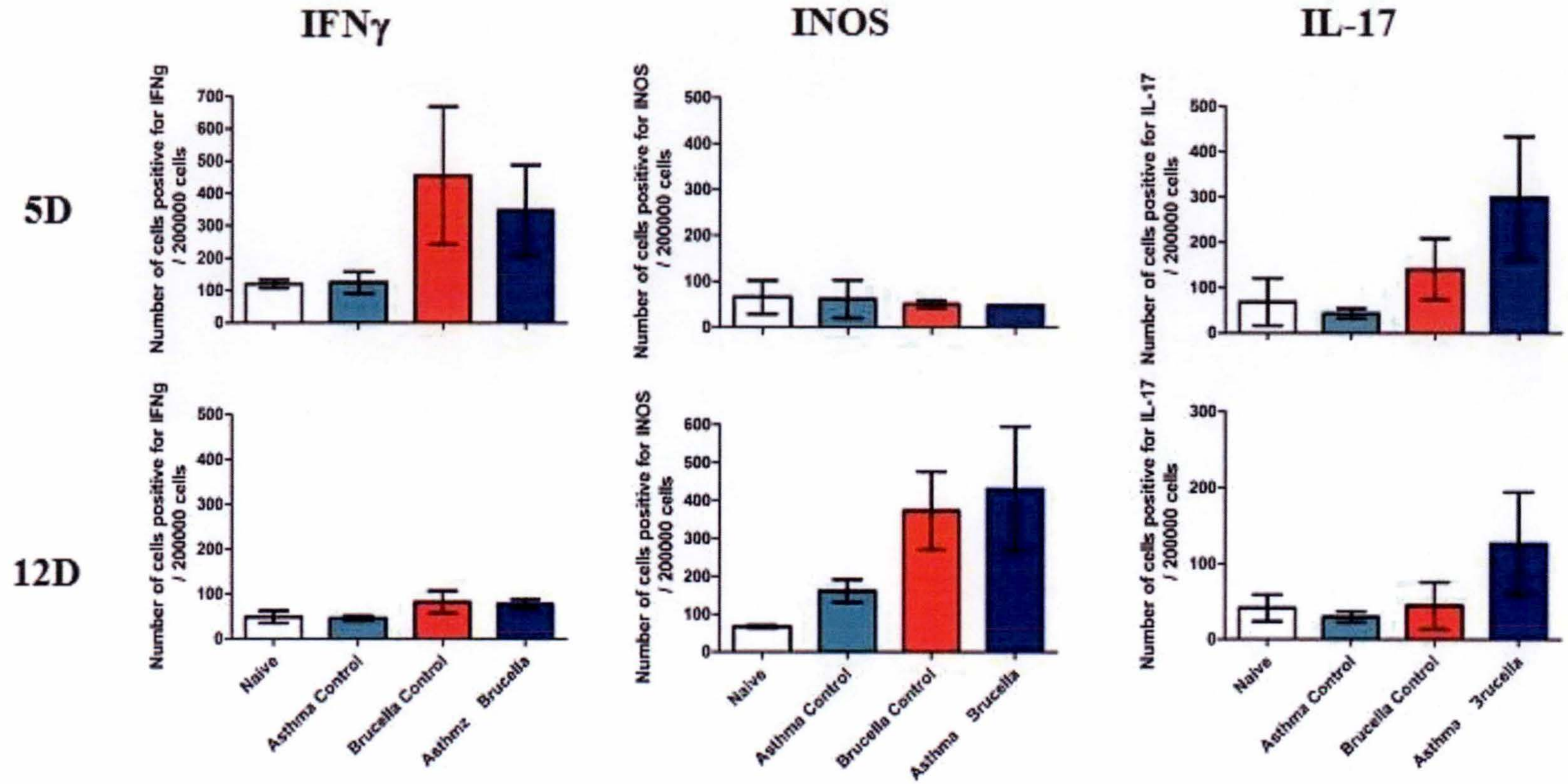


Figure 12: **Frequency of cell producing IFNγ, iNOS or IL-17 in BALB/c WT mice (Part 2)**

Flow cytometry analysis. Number of cells positive for IFNγ, iNOS or IL-17 on 200000 cells acquired. The frequency of positive cells was evaluated at 1, 5 and 12 days post infection. Naive and asthmatic mice received 3+1 PBS or HDM injections treatment respectively as previously described. The *Brucella* and the Asthma *Brucella* groups of mice were infected with $2x10^7$ CFU of *B. melitensis.* The columns represent the mean ± SD.

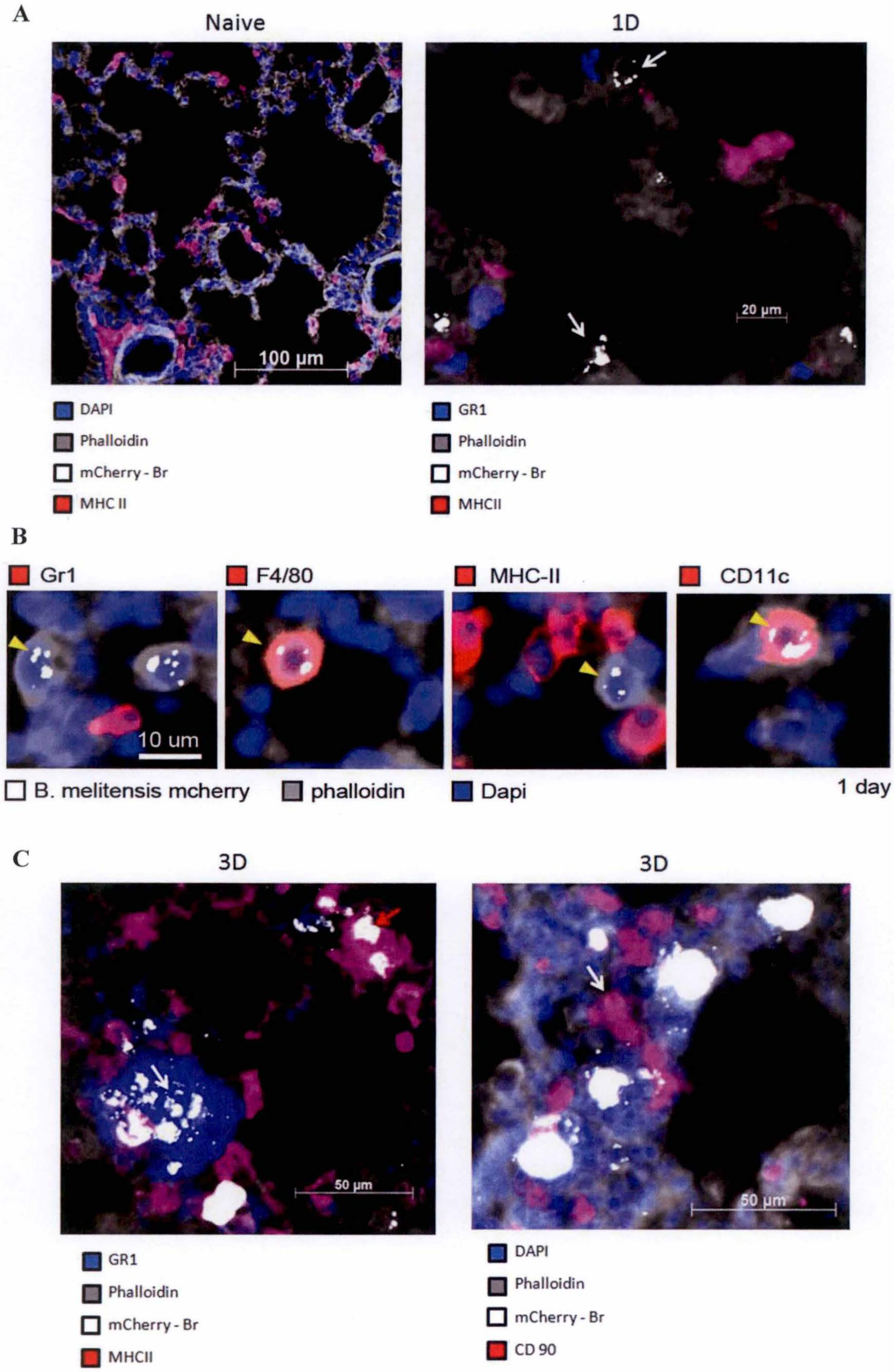


Figure 13: **Phenotype of infected cells (Part 1)**

Phenotype of infected cells and cell to cell spread

In an intranasal model of infections with *Brucella abortus*, it is only described that the alveolar macrophages are the primary cells infected by *Brucella* (Archambaud et al., 2010). So we first tried to characterized by immunohistofluorescence in the high infectious dose model, the recruitment of immune cells and the *Brucella melitensis* reservoirs cells in the lungs of infected mice before to comparing it with the asthmatic infected condition. This way, we will be able to test our second hypothesis: the T_H2 mediated inflammation of the allergic asthma alters the number and/ or the status of the reservoir cells in the lungs dampening their efficiency to respond to the infection and to kill the bacteria.

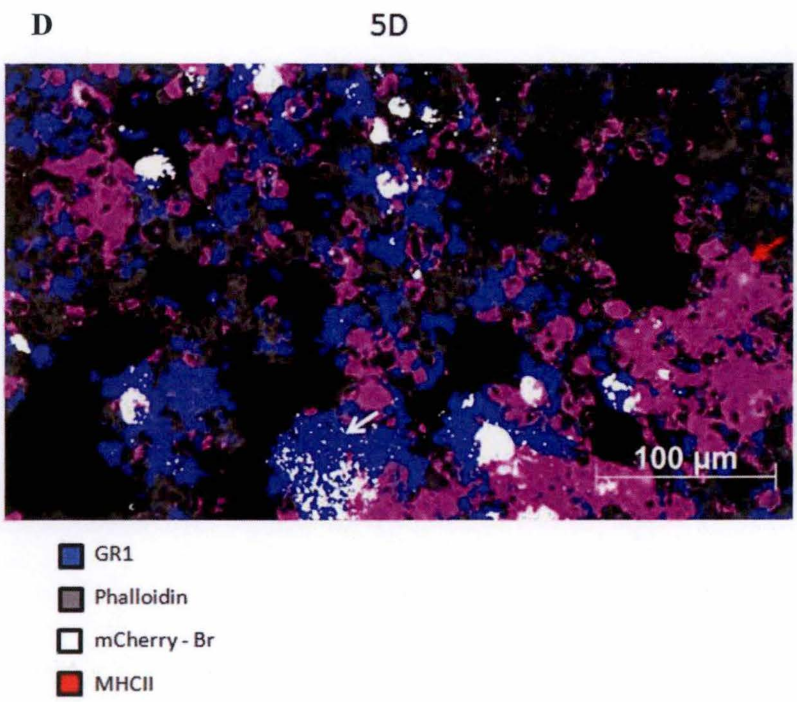
We confirmed that *Brucella* is mainly found inside alveolar macrophages (AM) at 1 day post infection. Infected cells display an AM phenotype as they are $GR1^-$ $F4/80^+$ $MHCII^-$ $CD11c^+$(Figure 13B). During the first 24 hours of infection, *Brucella* does not elicit the recruitment of immune cells. The state of the lung at 1 day post infection, is very similar compared to its state in naive mice (Figure 13A) (Figure 3). At 3 days post infection, the status of the lung has drastically changed. There is an general inflammation. The AMs are full of *Brucella* compared to day 1. There is an important increase of T cells ($CD90^+$) at the proximity of the infected cells. The infected AMs are overwhelmed by a massive recruitment of granulocytes ($GR1^+$). Preliminary data of flow cytometry analysis confirmed that there is an important recruitment of neutrophils in infected mice (Figure 14). We observed spreading from the infected alveolar macrophages to these agglutinated granulocytes (Figure 13C). At 5 days post infection, the inflammation is augmented and the dissemination to granulocytes is more frequent than at 3 days (Figure 13D). These results suggest that alveolar macrophages as the primary infected cells play an important role in the dissemination to other cell type, mainly neutrophils.

Aveolar macrophages, a replicative niche for *Brucella*

Through confocal analysis of lung sections, we confirmed that *Brucella melitensis* mCherry was well located inside the alveolar macrophages. We also characterized the growth curve of *Brucella* inside the alveolar macrophages. To perform this, we counted the number of *Brucella* per AM at different timepoints during the early course of infection (at 10 minutes, 2 hours, 1, 3 and 5 days post infection). The growth curve of *Brucella* defines a sigmoid curve with a plateau phase between 3 and 5 days post infection (Figure 15). At 10 minutes post infection, the only infected cells found are AMs with a maximum of 1 *Brucella* per cell. The *Brucella* load increases slightly during the next 2 hours as we can found an average of 3 to 4 bacteria per cell. The replication of *Brucella* inside the AMs increases importantly between 1 and 3 days. Then the replication rate of *Brucella* stabilizes at 3 days post infection and the number of *Brucella* per AMs was maximal at 5 day with an average of 125 *Brucella* per AMs. These results confirm that alveolar macrophages are important niche cells for *Brucella* supporting its replication.

The preliminary data confronting the *Brucella* infected and the asthmatic infected mice showed no differences in the growth curve of Brucella in the alveolar macrophages (Data not shown). If this results is confirmed we will investigate if the allergic asthma augments the number of the alveolar macrophages in the asthmatic condition.

D



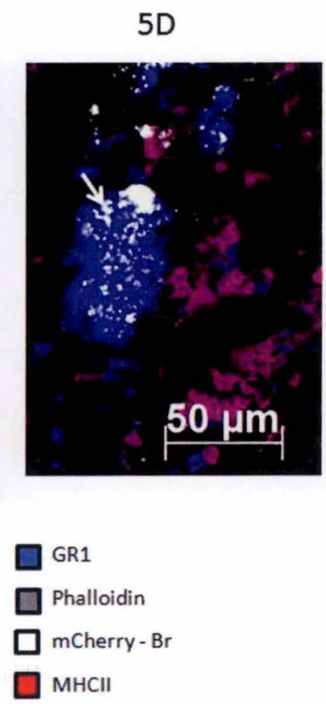


Figure 13: **Phenotype of infected cells (Part 2)**

Immunohistofluorescence analysis of DAPI (DNA), GR1 (Ly-6G), MHCII, CD90, CD11c and F4/80 expressing cells and phalloidin (actin) expression in the lungs. BALB/c WT mice were infected with $2x10^7$ CFU of *B. melitensis* 16M mCherry and compared with naive mice that received a RPMI injection. The lungs were collected at 1 (A & B), 3 (C) and 5 (D) post infection and examined by immunohistofluorescence. A. White arrows point alveolar macrophages. B. Phenotype of alveolar macrophages C. Red arrows point $MHCII^+$ AM. White arrows point recruitment of cells. D. Red arrow points $MHCII^+$ cells. White arrow points dissemination to $GR1^+$ cells. Panels beside the picture represent the markers used and associated color. Each image chosen is representative.

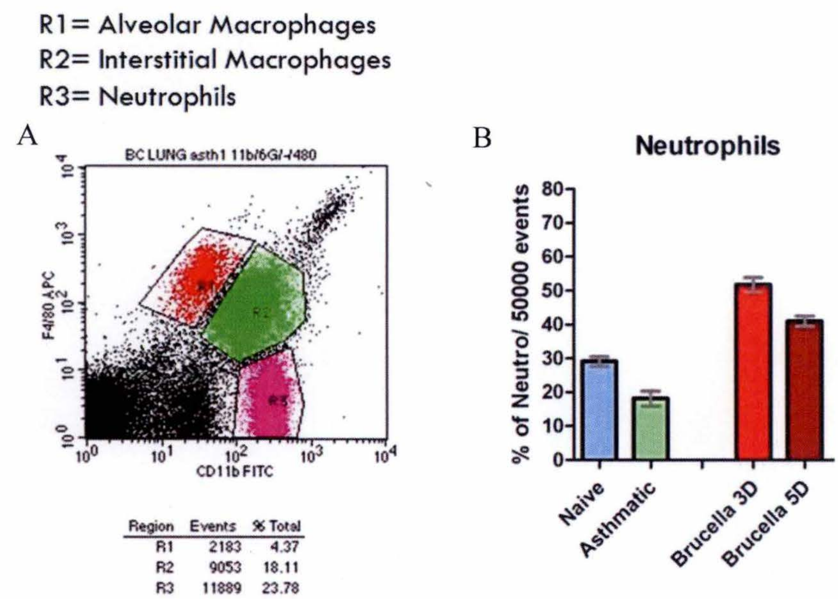


Figure 14: **The infection by Brucella induce the recruitment of neutrophils.**
Flow cytometry analysis. A. Representative plots used to gate alveolar macrophages, interstitial macrophages and neutrophils. B. Flow cytometry analysis of the frequency of neutrophils in the lung on 50000 events evaluated at 3 and 5 days post infection. Naive and asthmatic mice received 3 PBS or HDM injections treatment respectively as previously described. The *Brucella* and the Asthma *Brucella* groups of mice were infected with $2x10^7$ CFU of *B. melitensis.* The columns represent the mean ± SD. (N=3) Representative of one experiment.

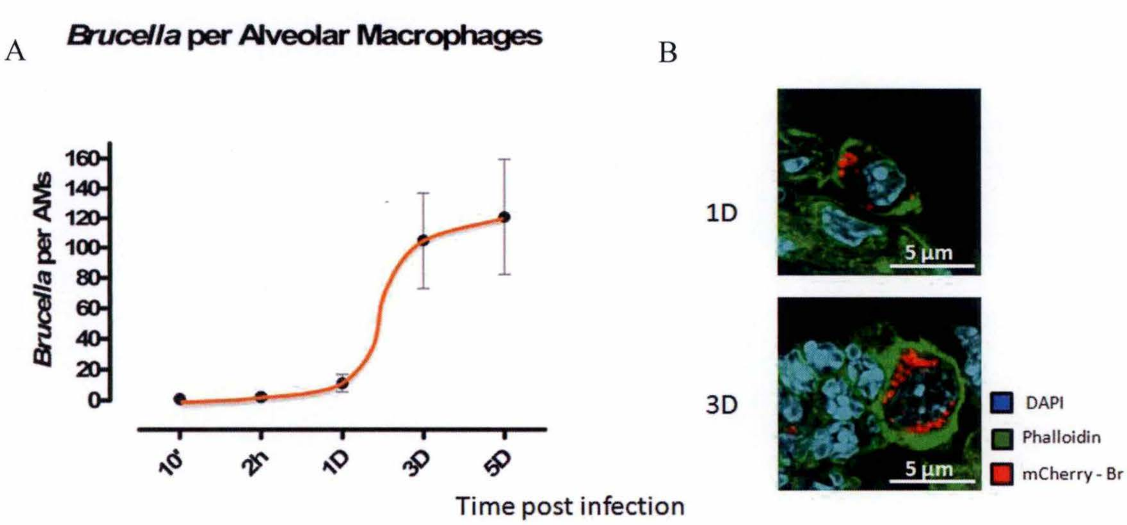


Figure 15: **Aveolar Macrophages, a replicative niche for *Brucella***
A. Growth curve of *B. melitensis* in alveolar macrophages at 10 minutes, 2 hours, 1, 3 and 5 days post infection. Points represent mean ± SD. Brucella was counted in at least 100 infected alveolar macrophages per condition in the lung of different infected mice. B. Confocal analysis of *Brucella* mCherry, DAPI (DNA) expressing cells and phalloidin (actin) expression in the lungs. Infected alveolar macrophage at 1 and 3 days post infection. Panels next the picture represent the markers used and associated color. Each image chosen is representative.

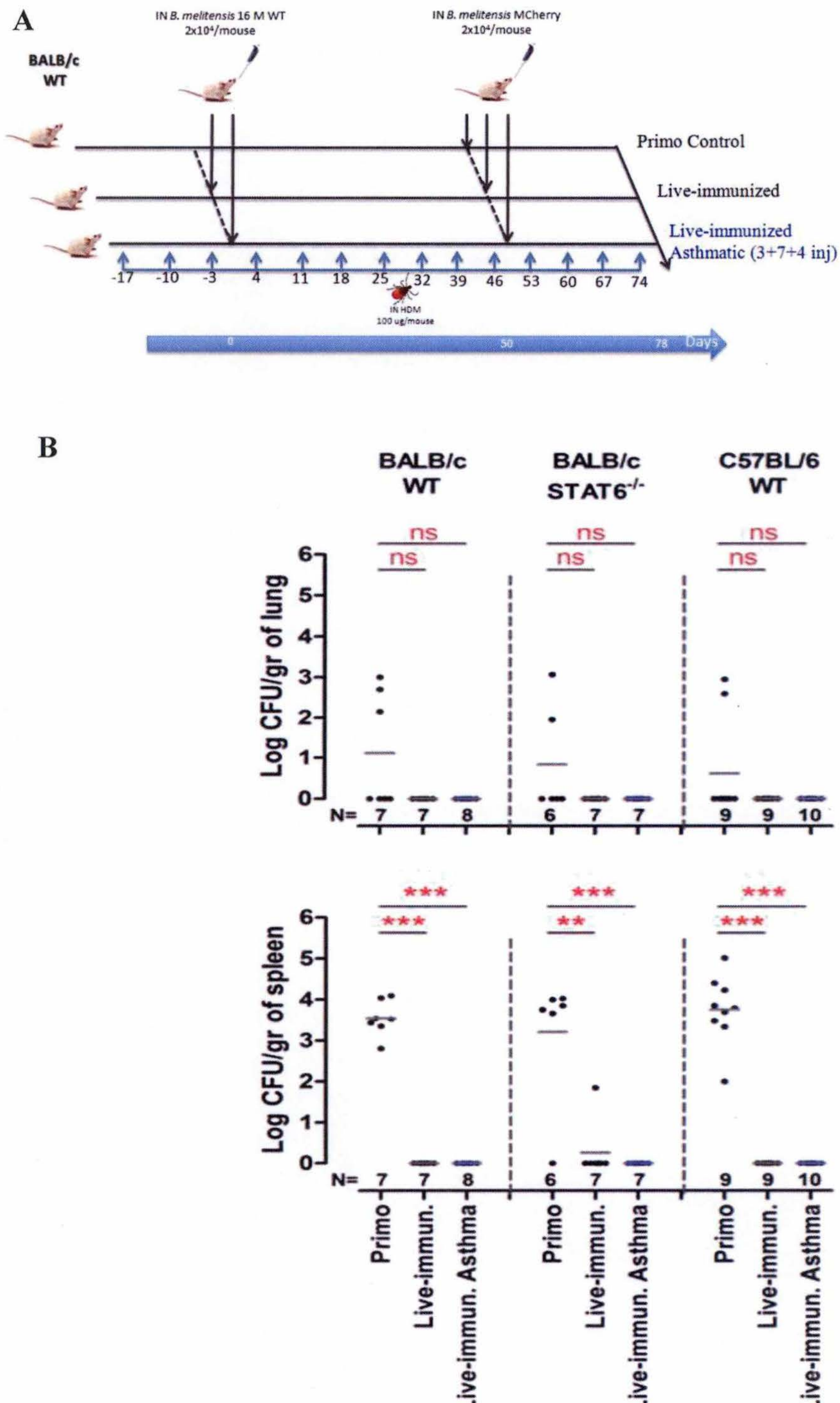


Figure 16: **Allergic asthma does not impair the immunization against *Brucella***

A. Scheme representing the steps and the condition of the experimentation B. CFU counts analysis. The data represent the number of CFU per gram of organ. The *Brucella* load was evaluated in the spleen and the lung at 78 days post first infection. Horizontal gray bars are the mean. These results are from one experiment (*P < 0.05, **P < 0.01 and ***P < 0.001). N denotes the number of mice used for each condition.

IV. Impact of allergic asthma on the development of protective immune memory against *Brucella*

Following intranasal infection, wild type mice acquired a better control of *Brucella* growth during a challenge infection (Delphine Hanot Mambres thesis). This protection has been related to the development of a $CD4^+$ T cells dependent protective Th1 memory response (Vitry et al., 2014).

As we have observed that asthma-induced Th2 environment impairs the control *Brucella melitensis* in the lungs, we investigated if asthma could also negatively affect the development of a protective immune memory resulting from *Brucella* infection. BALB/c WT, BALB/c STAT-$6^{-/-}$ and C57BL/6 WT received control or HDM treatment before and during *Brucella* infection (see Figure 16.A). All group of mice where challenged intranasally with $2x10^4$ CFU of mCherry-expressing *Brucella melitensis* strain and sacrificed 28 days later to compared the CFU level in the lungs and the spleen. 50 days post infection, the fluorescence of the challenge strain allow to discriminate between CFU resulting from primo infection and challenge infection.

The results presented Figure 16.B clearly showed that all immunized mice groups similarly eliminated the challenged *Brucella* strain from both the lungs and the spleen at 28 days post challenge infection. Independently of the sensibility of mice to HDM treatment, all group of mice appear able to control *Brucella* infection suggesting that HDM treatment has not interfered with the development of protective response. Note that we can't affirm that the spleen and the lungs are completely cleared due to the threshold limit of our CFU analysis (Detection threshold = 10 CFU). As expected, in our model, *Brucella* persists in the spleen and the lungs of primo infected mice at 28 days post infection.

Taken globally, these results demonstrate that allergic asthma has no negative impact on development of a protective immune memory against *Brucella* in our experimental model. However, these results are from only one experiment and need to be confirmed.

DISCUSSION

Brucella spp. are furtive bacteria that have evolved mechanisms to evade the immune system and to establish its long term persistence in host. Avoiding recognition, the bacteria do not elicit strong inflammation. *Brucella* hides from the immune system to ensure its successful infection. This stealthy bacteria through its numerous effectors has the capacity to infect professional phagocytes and to interfere with their killing activities (Martirosyan and Gorvel, 2013). However, in contrast to other causative agents of granulomatous diseases such as *Mycobacterium tuberculosis,* the agent of tuberculosis (Ernst, 2012), *Brucella* is not able to persist at high levels in the lungs. A previous study (Hanot Mambres et al., in preparation) suggests that T_H1 and T_H17 mediated immune responses are implicated in the control of *Brucella* in the lungs.

Models of cross-pathology have gained increased interest these recent years (Stelekati and Wherry, 2012). They have been used as a tool to unveil actors and mechanisms implicated in the immunological responses to pathogens. In this study, we analyzed the impact of allergic asthma on the ability of mice to control intranasal *Brucella melitensis* infection.

In a preliminary work, we confirmed in our laboratory conditions, the results of previous studies (Hammad et al., 2009; Marichal et al., 2010) showing that repeated intranasal house dust mites (HDM) injections in mice induce a T_H2 allergic asthma phenotype similar to the trait found in humans. We observed that the main features of allergic asthma were well present in our model such as an augmentation of the total IgE titers, massive infiltrations of cells notably granulocytes (eosinophils and neutrophils) and mast cells at the proximity of the conducting airways, goblet cells hyperactivation and associated increased mucus secretion. The HDM treatment is also more physiologic and representative of what happens in nature. Interestingly, the repeated HDM exposures do not elicit tolerization to the allergen as it is the case with asthma model induced by intraperitoneal injections of ovalbumin (Bates et al., 2009). Thus, this model appears more representative of what happens in natural conditions during asthma sensitization. Our results also showed that C57BL/6 mice develop a less marked allergic asthma when compared to BALB/c mice, as demonstrated by IgE measurements. This difference between C57BL/6 and BALB/c mice strains could be explained by their genetic bias to mount preferentially a T_H1 or T_H2 immunity respectively (Paul and Zhu, 2012). Thus, we have chosen to mainly analyze the cross pathology model in BALB/c WT mice.

In the first part of our work, we demonstrated that the HDM-induced allergic asthma phenotype compromises the ability of mice to control the intranasal infection with *Brucella melitensis*. The *Brucella* burden was significantly augmented in the lungs of asthmatic mice. This impact seems restricted to the lung compartment, as it did not affect the *Brucella* load neither in the spleen nor in the liver. However, it would be interesting to investigate the mediastinal draining lymph nodes. This phenomenon is strictly dependent of allergic asthma-induced T_H2 environment as it is absent in STAT-6 deficient mice that are not able to develop a T_H2 response. In an attempt to optimize the impact of asthma, we increased to six the number of challenges before the infection. The results showed no significant effect on the *Brucella* load compared with the previous model of HDM treatment. This suggests that the preexisting allergic asthma phenotype induced by only 3 HDM injections before the infection was already optimal to alter the control of *Brucella*. However, the addition of only one HDM challenge during the course of infection was sufficient to increase the *Brucella* burden in the lungs of the asthmatic mice. We also observed that HDM-induced allergic asthma enhances the capacity of *Brucella* to persist in the lungs for a longer time and the effect is proportional to the number of HDM challenge during the course of infection.

The infection by *B. melitensis* stimulates the induction of a T_H1 immune response which could dampen the T_H2 response of the allergic asthma (Arora et al., 2010). As an illustration, the unlipidated outer membrane protein (omp) 16 of *Brucella* has been shown to prevent the induction of T_H2 allergic disorder through the induction of a T_H1 immunity (Ibañez et al., 2013). Thus, the HDM challenge during the infection could increase the impact of asthma by the maintenance of the T_H2 environment. T_H2 mediated inflammatory disorders like allergic asthma promotes the polarization of macrophages into M2 macrophages (Habibzay et al., 2013). The additional HDM challenge could also maintain the polarization of the alveolar macrophages as they can switch from a M2 to M1 state depending on the cytokinic environment (Guilliams et al., 2013). Some indirect experiments have suggested that M2 macrophages could be a preferential niche cells for *Brucella* (Xavier et al., 2013a).

In the second part of our work, we tried to elucidate the mechanism by which HDM-induced allergic asthma affects the growth and persistence of *Brucella* in the lungs.

First hypothesis: allergic asthma inhibits T_H1 protective immune response against *Brucella*:

It is well known that the allergic asthma-induced T_H2 response is able to inhibit the development of an anti bacterial T_H1 response (Barnes, 2008). For example, chronic HDM exposures inhibit the recognition of the bacteria *Streptococcus pneumoniae* by the TLR which impaired the host immunity to control the pathogen (Habibzay et al., 2012). So we hypothesized that the preexisting T_H2 environment induced by the allergic asthma inhibits the induction of the T_H1 immune response well described to control *Brucella* infection (Copin et al., 2007). In order to test this hypothesis, we have analyzed the ability of allergic asthma to affect *Brucella* control in IL-12p40 deficient mice that display a reduce ability T_H1 and T_H17 protective immune responses against *Brucella*. Surprisingly, we have observed in these mice that HDM sensitization protocol conserve its capacity to strongly affect the control of *Brucella* in the lungs of IL-12p40 deficient mice. This result demonstrates that allergic asthma favors *Brucella* growth in the lungs independently of the inhibition of T_H1 and T_H17 responses. In agreement, we observed by flow cytometry that the production of IFN-γ, INOS (T_H1) and IL-17 (T_H17) in the lungs of asthmatic infected mice is enhanced when compared to infected control mice. However, the augmentation of these cytokines did not improve the control of the infection.

A recent cross infection study (Potian et al., 2011) of the parasite *Nippostrongylus braziliensis* with *Mycobacterium tuberculosis* (Mtb) showed similar results. The T_H2 context induced by *N. braziliensis* increased the susceptibility of the infected mice to a secondary infection with Mtb. The loss of control of the Mtb infection was not due to the inhibition of the T_H1 response as both T_H1 and T_H2 effector cells were still recruited in the lungs. The increased Mtb burden appeared to be the result of an accumulation of M2 macrophages mediated by the IL-4 pathway. This study showed that the interactions between T_H responses can be far more complex than predicted by the classical T_H1/ T_H2 paradigm.

Second hypothesis: allergic asthma alters the number and/ or the polarization of the *Brucella* reservoir cells in the lungs:

The innate immunological environment polarizes the $CD4^+$ T cells to drive the T_H immune responses adapted to pathogens control. In turn, T_H1 and T_H2 cytokines also, drive the polarization of macrophages (Muraille et al., 2014). T_H2 mediated inflammatory disorders like allergic asthma promotes the polarization of macrophages into M2a macrophages. These M2 macrophages have a reduced efficiency to kill intracellular bacteria such as *Mycobacterium tuberculosis* (Habibzay et al., 2013). In the *Brucella* model, it has been shown that pharmacological treatment inducing the differentiation of M2a macrophages in mice favor the growth of *Brucella* in the spleen of infected mice. In vitro, *Brucella* displays a higher growth in M2 macrophages when compared to M1 macrophages. This phenomenon has been correlated to the higher glucose metabolism in M2 macrophages supporting the replication and persistence of *Brucella* (Xavier et al., 2013a).

We first tried to characterize the recruitment of immune cells and the *Brucella melitensis* reservoirs cells in the lungs of infected mice before to compare it with the asthmatic infected condition. This way, we will be able to test our second hypothesis. Consistently with a previous study with *Brucella abortus* (Archambaud et al., 2010), we observed rapid infection of the alveolar macrophages (AMs) by *Brucella melitensis*. The infection seems silent and does not elicit detectable inflammatory response until 3 days post infection. This furtive strategy enables *Brucella* to reach its replicative plateau phase inside the AMs before the recruitment of immune cells notably neutrophils. The outer membrane protein (omp) 19 of *Brucella* has been shown to induce the recruitment and the activation of neutrophils associated with an increase survival of these cells (Zwerdling et al., 2009). The calling of neutrophils could be a strategy for *Brucella* as we observed by microscopy analysis the dissemination of *Brucella* from the infected AMs to the agglutinated neutrophils. One study showed in vitro, that *Brucella abortus* was capable of infecting and surviving inside neutrophils (Kreutzer et al., 1979).[4] In mice, the infiltration of neutrophils is detrimental for the control of the infection as it reduces the T_H1 immune response against *Brucella* (Barquero-Calvo et al., 2013). In the present work, we described the different phases of the infection by *Brucella* in the lungs. In later work, these results will be compared with the asthmatic condition to observe what is similar and to highlight differences. We will focus on the infected cells, the replication rate of *Brucella* in the reservoir cells and the recruitment of immune cells.

Potential implications for the public health

Our results suggest that allergic asthma is an important risk factor for brucellosis. The T_H2 induced airway inflammation increases the susceptibility to an intransal inffection by *Brucella melitensis*. The loss of control leads to an outgrowth of the bacteria in the lung of infected mice which is associated with an exacerbation of the inflammatory response. This vicious cycle has been described with other bacterial pathogen such as *Streptococcus pneumoniae* and *Staphylococcus aureus* to result in a significant increased morbidity and poor outcome for the patients (Habibzay et al., 2013).

Asthmatic individuals have an altered microbiota with an augmentation of pathogenic proteobacteria carriage notably *Haemophilus influenzae* (Hilty et al., 2010). This modification of the microbiota could be explained by the sustained mucus secretion and accumulation in the asthmatic airways. With an enhanced viscosity and density in allergic asthma condition,

[4] Note that the experiments of (Kreutzer et al., 1979; Zwerdling et al., 2009) has been done in another model. As transposition from one model to another is tricky, these results are taken with cautious.

the mucus is shown to hide bacteria from the immune system (Wilson et al., 1996). The usual long-term treatment for asthma relies on the use of glucocorticoids. The therapy has an anti-inflammatory and an immunosuppressive action which renders the patients even more susceptible to secondary infection (Edwards et al., 2012). Our experiments on mice with a T_H1 and a T_H17 depletion context showed similar results with a complete uncontrolled bacterial growth of *Brucella melitensis* in the lungs of the asthmatic mice. Novel therapeutic approaches with the use of macrolide seem more indicated for asthmatic people exposed to dangerous bacterial pathogens such as *Brucella*. Ketomacrolides have been shown to reduce asthmatic symptoms in conjunction with their antibiotic activity (Johnston et al., 2006).

Impact of allergic asthma on the development of protective immune memory against *Brucella*

There is accumulating evidence that the T_H2 immune response induced by helminths could lower the efficiency of pneumococcal and Bacillus of Calmette and Guérin (BCG) vaccines (Apiwattanakul et al., 2014; Elias et al., 2006b). The modulation of the host immunity by the helminths decreases the control and subsequent induction of the memory (McSorley et al., 2013). In addition, the T_H2 associated excessive mucus secretion could also reduce the efficiency of the vaccine uptake by the host (van Riet et al., 2007). As we have demonstrated the T_H2 immune response induced by allergic asthma impairs the control of the infection by *Brucella melitensis* similarly of the helminths *N. braziliensis* with MtB, we wondered if allergic asthma could negatively impact the immunization against *Brucella*. It would be of great relevance for the public health to investigate if asthma could impairs vaccines against dangerous pathogens such as MtB, *Brucella* and *Streptococcus pneumoniae*. Asthmatic people are an important target population for vaccinations as they are generally more susceptible to be infected by a wide range of pathogens and so need enhanced immunity (Edwards et al., 2012).

A previous study (Vitry et al., 2014b) from our group characterized the immune cells necessary for the generation of an efficient immune memory response against *Brucella* in a intraperitoneal infection model. They showed a reduced efficiency of BALB/c WT mice compared to C57BL/6 WT mice to develop a protective memory immune response against *Brucella*. The ability of BALB/c was restored in STAT6 deficient mice. Through our model, we have investigated if the IL-4 pathway mediated T_H2 immune response could impact the induction of a protective immunity against *Brucella* to have more insights on the immunization mechanism of *Brucella*. We showed more clear-cut results. In our model, all mice strains were able to eliminate the challenge strain. We have demonstrated in our experimental model that the T_H2 immunity of allergic asthma has no negative impact on the development of a protective immune memory against *Brucella* in our experimental model. Our results suggest that allergic asthma does not affect the development of protective immune response against all bacteria. Vaccination for asthmatic people could be effective against *Brucella* with live immunization. However, in the case of *Brucella*, the available live attenuated vaccines remain not safe for humans (Godfroid et al., 2011).

Future perspectives

We have characterized the *Brucella* reservoir cells and the recruitment immune cells upon *Brucella* infection at an early time through flow cytometry and immunohistofluorescence analysis. We will compare this data to the asthmatic infected condition. We will monitor the number of alveolar macrophages in the lungs during the course of infection. As an increased number of alveolar macrophages is frequently observed in HDM sensitizations model (Maus et al., 2006), this could enhance the capacity of *Brucella* to establish an efficient infection in the lungs. The replication rate of *Brucella* could also be accelerated in the AMs due to their M2 polarization under a T_H2 environment (Xavier et al., 2013a). We will also evaluate the ratio of M1 and M2 macrophages with specific markers as it has been shown to play an important role in a previous cross-infection study (Potian et al., 2011). The investigation of the mediastinal draining lymph nodes could improve our understanding on the dissemination mechanism used by *Brucella*. The use of mice genetically deficient for cytokine and chemokine implicated in the regulation of the chemotaxis of neutrophils (Eash et al., 2010; Smart and Casale, 1994) may also help us to sort out the role of neutrophils in the impact of asthma. In addition, we will confirm by confocal analysis that *Brucella* are well inside the neutrophils.

It has been shown that IL-10 has a negative impact on the control of *Brucella* (Xavier et al., 2013b). We will evaluate the importance of this cytokine in the impact of asthma by the use of IL-10 deficient mice.

In collaboration with the Olivier Denis research group, we will confirm the impact of allergic asthma with another model using the mold *Alternaria alternata* shown to induce a TH1 or TH2 immune response depending on the extracts used (Denis et al., 2007). We will also confirm our results on the immune memory against *Brucella*.

A lot of studies have described the impact of bacterial infections on the pathogenesis and the exacerbation of asthma (Habibzay et al., 2013). It could also be interesting to reverse our cross pathology model and study the impact of *Brucella* infections on the development of allergic asthma. It has been shown that one outer membrane protein of *Brucella* can prevent the induction of allergic bowel disorder (Ibañez et al., 2013).

MATERIALS & METHODS

Mice and ethics statements. C57BL/6 wild type (WT), C57BL/6 IL10$^{-/-}$, BALB/c WT and BALB/c STAT6$^{-/-}$ mice were bred in an institutional conventional animal facility of the Free University of Brussels (ULB, Belgium) under the ethical regulation of the ULB. BALB/c and C57BL/6 WT mice were purchased from Harlan (Netherlands). All mice used were 6 to 8 weeks old. At least 8 mice per condition were used for statistical relevance. The protocol was approved by the Ethical Committee of Animal Experimentation of the University of Namur (Unamur, Belgium).

Mice infection and reagents. *Brucella melitensis* strain 16 M WT (biotype 1, ATCC 23456) is Nal^{R}. MCherry *B. melitensis* strain 16 M stably express constitutively the protein mCherry and is Nal^{R} Kan^{R}. *B. melitensis* stock is stored at -80°C in 30% glycerol solution. One week prior the infection, the strain is taken from the stock solution and plated on 2YT medium plates. Fifteen hours before the infection the culture was grown in 10mL 2YT liquid medium (LB Agar [32g/L], Invitrogen ; Yeast Extract [5g/L], BD ; Peptone [6g/L], BD) at 37°C with shaking. The following day after the 15h incubation, the bacteria are harvested in the exponential growth phase. The culture is washed twice in RPMI (3500g for 10 minutes). Dilution to obtain appropriate infectious dose is based on OD measurement (600λ) (Copin et al., 2007).

$$\frac{\mathrm{OD} \times 3.10^9}{V_{ID}\,(\mathrm{mL})} = D$$

$$\frac{V_{TOT}}{D} = V_{Bact}$$

$$V_{TOT} = V_{Bact} + \mathrm{RPMI}\,(\mathrm{mL})$$

OD = 1 = 3.10^9 bacteria/mL
Vol_{ID} = Infectious dosis Volume (mL)
D = Dilution factor
V_{TOT} = Total Volume (mL)
V_{Bact} = Bacterial solution volume (mL)

Before the infection, mice are anesthetized by an intra peritoneal injection of a Xylazine XYL-M 2% (VMD - ref: 00115.02) (11,5µl)/Ketamine 1000 (Ceva - ref: 804119) (18µl) solution (400µl PBS/25g of mouse). Mice were infected with *Brucella melitensis* 16M WT or MCherry by intranasal inoculation (IN) with a dose of 2x10^4 or 2x10^7 CFU in 30µl of RPMI. Control mice received the same volume of PBS. The infectious doses were confirmed by plating serial dilutions of inoculum on 2YT medium plates (LB Agar [32g/L], Invitrogen ; Yeast Extract [5g/L], BD ; Peptone [6g/L], BD ; Agar [1g/L], BD).

After the infection, mice are euthanized at predetermined times. Different organs are harvested and different techniques are used depending on the goal pursued:

- The evaluation of the bacterial load of the organ by CFU on the spleen, liver and lung.

- The characterization of the infected cells and the recruitment of cells in the lung by immunohistofluorescence.

- Analysis and characterization of immune cells populations and cytokines produced in the lung by flow cytometry.

Mice euthanasia and dissection. Mice are euthanized by cervical dislocation. The mouse is set on its right side. Splash the fur with 70% ethanol for sterilization. Remove side of the skin and splash the peritoneum with 70% ethanol. Remove the peritoneum. Take the spleen (all) and the liver (one lobule). Cut the diaphragm and remove the rib cage. Take the lungs without tweezers to do no harm to the organ if histology needs to be proceeded.

Bacterial load count. Mice are euthanized by cervical dislocation. Lungs, liver and spleens are taken. These are homogenized in a sterile plastic bag by crushing through it and resuspended with 1 mL PBS-Triton X-100 (VWR ref: 28817.295, 0.1%). Successive serial 10 to 10 dilutions were realized in RPMI to obtain an optimal bacterial load to be plated onto 2YT Kanamycin (50µg/ml) medium plates. CFU were counted after an incubation of 5 days at 37°C.

Infectious Dosis $2x10^4$ CFU/ 30µl

Dilution		1D	5D	12D	28D	50D
Organs:	Spleen	0	0; -1	-1; -2	0; -1	0; -1
	Liver	0	0; -1	-1; -2	0; -1	0
	Lungs	0; -1	-1; -2; -3	-1; -2; -3	0; -1; -2	0

*if asthmatic mice

Infectious Dosis $2x10^7$ CFU/ 30µl

Dilution		3D	5D	12D
Organs:	Lungs	-2; -3	-2;-3; -4	-2; -3

*if asthmatic mice

Buffer.

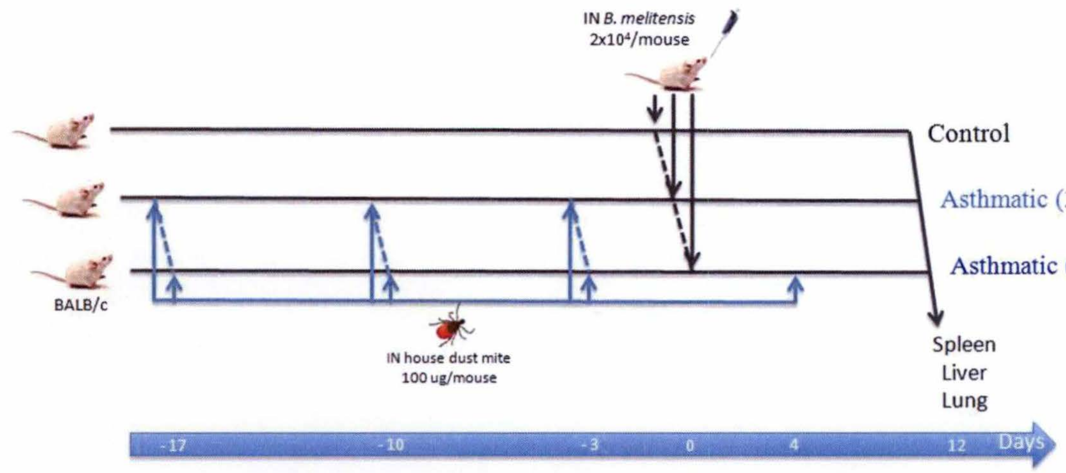
PBS10x

NaCl (80g/L), KCl (2g/L), Na_2HPO_4 (11.5g/L) and KH_2PO_4 (2.4g/L) dissolved under agitation in bidistillated water. Put to pH7.4. Store at room temperature.

PBS-0.1% Triton

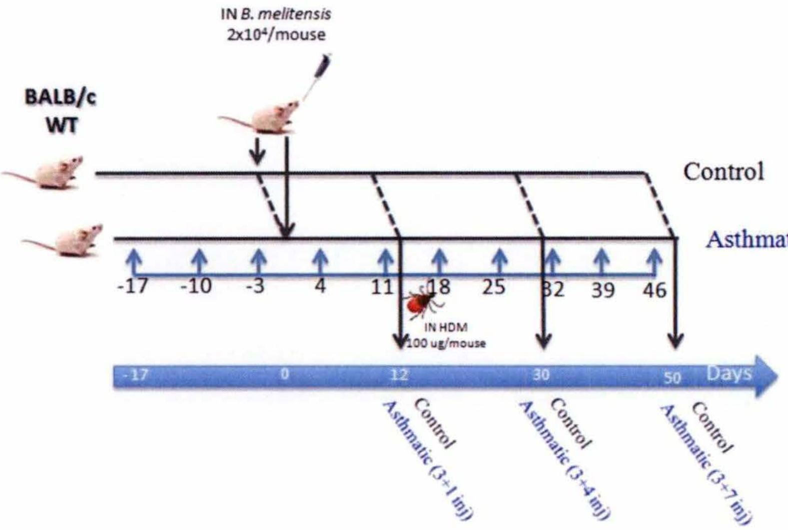
Dilute 500µl of stock Triton in 500mL of PBS. Watch out for the viscosity of the Triton. Autoclave the solution. Store at room temperature.

Induction of allergic asthma. Mice were gently anesthetized by inhalation of isoflurane (from Abbott laboratories (# No. B506)) and then received an intranasal injection of HDM extract (100µg in 50µL of PBS). Mice were sensitized on days -17, -10 and challenged with HDM on day -3 before the intranasal infection with *Brucella melitensis.* A rest time of one week is kept between two HDM injections. Control mice received the same volume of PBS by intranasal injection. The model of induction was obtained from Bureau F. from ULg (Marichal et al., 2010).



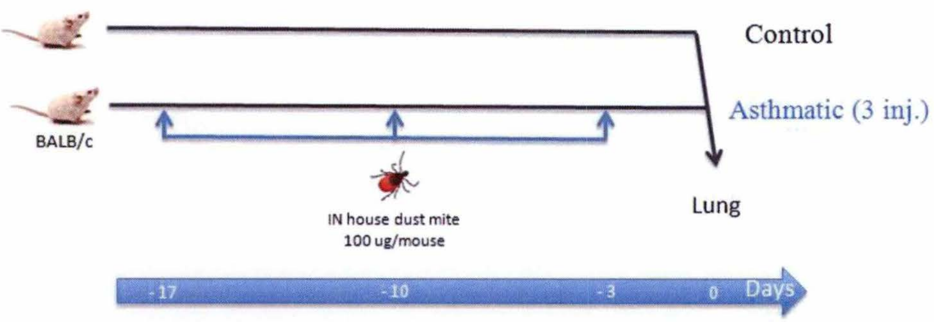
Reagents. Lyophilized house dust mites (HDM) (*Dermatophagoides farinae*) extract was from Greer Laboratories (Lenoir, NC; endotoxin contents: 0.84 ng/mg).

Chronic allergic asthma models. Asthmatic mice were challenged each week with HDM (as described above) after the intranasal infection with *B. melitensis* 16M since day 4 and until mice are euthanized. The name of the condition represents the number of HDM injections before and after the infection.



Evaluation time of the *Brucella* burden was determined on base of the course of the infection by *B. melitensis* in an intranasal model of infection.

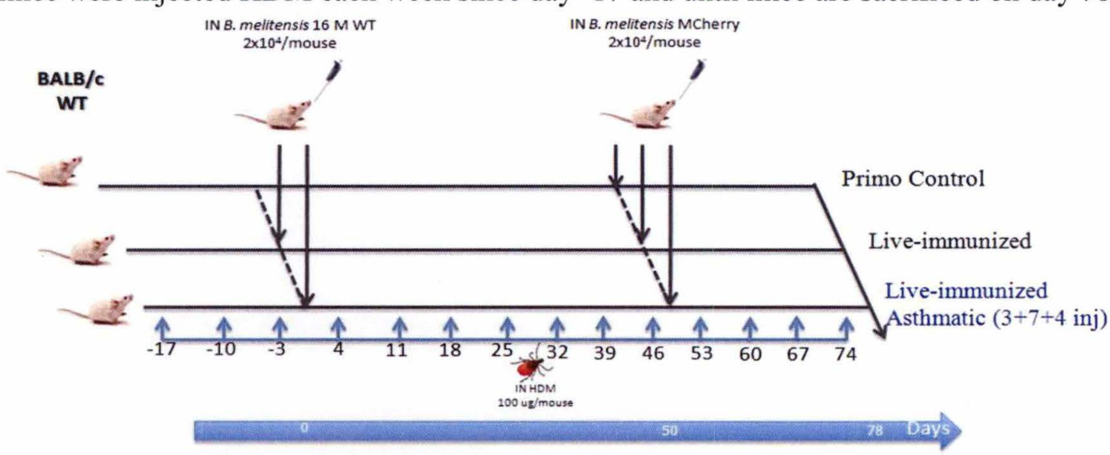
Control of the induction of allergic asthma. Mice were gently anesthetized by inhalation of isoflurane and then received an intranasal injection of HDM extract (100µg in 50µL of PBS) in the asthmatic condition or the same volume of PBS in control mice on days 0, 7 and 14. In both cases mice are killed on day 17 after the first HDM sensitization.



Determination of total IgE levels in blood serums. The end of the tail of the mouse is sectioned to induce a hemorrhage. The blood is taken with a heparinized capillary (Hirshmann – ref: 9100275). An average of 150µl of blood per mouse is taken. The serum is collected after centrifugation (10.000 rpm for 10 min) and then conserved at -20°C. The total IgE levels in the blood serums is measured by Enzyme-Linked Immunosorbent Assay (ELISA) by ELISA MAX™ kit purchased from Biolegend Inc. (San Diego, California, USA) following the manufacturer's protocol.

Total IgE ELISA test procedure. One day prior, add 100µl of diluted Capture antibody (1/200) in each well of a 96 well Nunc plate and incubate overnight at 4°C. Next day, wash 4 times and flick the plate. Add 200µl of Assay diluent to block non specific sites and incubate for 1h at RT with a 200rpm shaking. During the incubation, prepare diluted standards and samples in another 96 well plate. Wash 4 times and flick the plate. Transfer diluted standards and samples to the plate and incubate for 2h at RT with a 200rpm shaking. Wash 4 times and flick the plate. Add 100µl of diluted detection antibody (1/200) and incubate 1h at RT with shaking. Wash 4 times and flick the plate. Add 100µl of diluted Avidin-HRP (1/1000) and incubate 30' at RT with shaking. Wash 4 times and flick the plate. Add 100µl of TMB substrate mix A (1/2) + B (1/2) and incubate in the dark for 20min. Stop the reaction by adding 100µl of 2N H_2SO_4. Read the absorbance of the plate at 450nm – 570nm.

Immune memory test against *Brucella*. On day 0, mice were infected as described above with *Brucella melitensis* 16M WT by IN with a dose of $2x10^4$ in 30µl of RPMI. Control animals were injected the same volume of PBS. 50 days after, mice were challenged with *B. melitensis* 16M MCherry by IN with a dose of $2x10^4$ in 30µl of RPMI. On day 78, the blood of the mouse was taken. Mice were then euthanized for bacterial count. The infectious doses were confirmed by plating serial dilutions of inoculum on 2YT medium plates. Asthmatic mice were injected HDM each week since day -17 and until mice are sacrificed on day 78.



Histology Protocols

Lung Histology.

Mice are euthanized by cervical dislocation and lungs are taken. Lungs are fixed in a 4% formol 1% acetic acid solution over night. Impregnation of the organs in paraffin is done via a Sakura-Tissue Tek – Vip automate.

Dehydration: Absolute Methanol during 45min. at 35°C (4 cycle).
Toluene: 100% Toluene during 45min. at 35°C (2 cycle)
100% Toluene during 60min. at 35°C (2 cycle).
Paraffin: Paraffin during 60min. at 60°C (4 cycle).

Organs are embedded in paraffin cassette with 60°C paraffin then cooled down until solidification. Blocs are unmolded and cleaned by 25µm sections. After a 3 to 15 hour treatment with Molifex^{R}, the blocs are ready to be cut in 6µm sections. Ruban sections are kept in an ad hoc case. Can be conserved at 4°C. Sections are cut from the ruban and put on slice with a humid lance. The slice is put on a 53°C plate. The heat unfolds the section. Water in excess is eliminated. The slice put vertically on a metallic rack is dried in a blower at 48°C. Then can be conserved several months at RT.

Before any staining, slices are unparaffined and dipped in the following solutions. Dip in 100% Toluene for 2x3 min. Dip in 100% Methanol for 2x3 min. Dip in 70% Methanol for 2x3 min. Rinse with tap water for 10 min to whiten the slides.

Topographic Stainings

Hemalun – Erythrosine – Saffron staining

Staining Procedure. Dip the slides in pools of the following solutions under constant agitation. Dip in Hemalun solution (Hematein [3g/L] in Alun potassium saturated solution heated up to boiling with 2% glacial acetic acid) for 7min. Rinse with tap water. Dip in Ethanol-HCl (0.4% steaming HCl in Absolute ethanol) for 1min. Dip in tap water with light alkaline pH for 10min. Dip in Erythrosine (Erythrosine [7.5g/L] in tap water with 35% Formol [10drops/L]) for 6min. Make a quick rinse with tap water. Dip in 70% ethanol for 1min. Dip in 100% ethanol (3x2min). Dip in Saffron (Gratinais' Saffron [7.5g/L] in 100% ethanol. Heat until boiling with refrigerate column.) solution for 8min. Make 3 quick rinses in 100% ethanol. Make 3 quick rinses in 100% toluene. Assemble the cover slip on the slide with DPX.

Trichromic Light Green staining

Staining Procedure. Dip the slides in pools of the following solutions under constant agitation. Dip in Hematoxylin (Gill's Hematoxylin III [8g/L], Aluminum sulfate [70,4g/L], Sodium iodide [0,8g/L] in distillated water with 2% acid acetic) for 7min. Make a quick rinse in tap water. Dip in Ethanol-HCl (0.4% steaming HCl in Absolute ethanol) for 1min. Dip in tap water for 10 min. Dip in Ponceau-Fuschin (Fuschin acid [0.1g/L], Xylidin Ponceau [0.1g/L] in 1% acetified water) solution for 4min. Make a quick rinse in tap water. Dip in 5% Phosphotungstic acid for 3min. Dip in Light green (Light green Yellowish [0.1g/L] in 1% acetified water) solution for 6min. Make 3 rinses of 1min in 1% acetified water. Assemble the cover slip on the slide with DPX.

Histochemical stainings

Periodic Acid Schiff (PAS): put sugar in evidence

The periodic acid ($HIO_4.2H_2O$) oxide the sugars and transform glycols into aldehydes.

R-CHOH-CHOH-R' => R-CHO+ R'-CHO + H_2O

The Schiff reagents reacts with aldehydes and generates a colored precipitate in presence of sulfite ions.

Staining Procedure. Dip the slides in pools of the following solutions under constant agitation. Rinse in H_2O d. for 2x1min. Dip in 1% HIO_4 for 20min. Rinse in H_2O (2x1min). Sponge the slides and dip them in the Schiff solution for 20min. Dip in producing ions SO_3^{-} solution for 3x2min. Rinse in H_2O d. for 2x1min. Rinse in tap water. Make a second staining Hemalun – Safran as done in HES but skip the erythrosine step! Assemble the cover slip on the slide with DPX. Make a positive and a negative control.

Toluidine Blue staining: put metachromasy in evidence (for mastocytes)

This staining is used to turn the granulates of the mastocytes (histamine – heparin – serotonin) into a red – fuschia color.

Staining Procedure. Dip the slides in pools of the following solutions under constant agitation. Rinse in H_2O d. for 2x1min. Dip in 0.1% Toluidine Blue (diluted in acetate buffer at pH 4,05 precisely) for 15min. Rinse with energy in acetate buffer (20%[0.2M] sodium acetate + 80% [0.2M] acetic acid and set to pH 4,05) for 10sec. Sponge the slides and immerge them in tertiary butyl alcohol with shakings for 15 to 30sec. Assemble the cover slip on the slide with DPX.

Lung Immunohistology.

Mice are euthanized by cervical dislocation and lungs are taken. Lungs are fixed in paraformaldehyde solution 2% at room temperature (RT) for 1h30 to 2 hours at null pressure. Lungs are washed twice in PBS and then submerged in PBS sucrose 20% (dissolved in PBS, VWR - ref: 27483294) solution overnight at 4 °C. Sponge the organ to remove sucrose. Prepare disposable plastic cryomolds (VWR, ref: 25608-92) and full it with OCT (Tisssue-Tek – ref: 4583). Dispose obliquely the lungs at the bottom of the cryomolds and incubate for 10min at RT. The organs are then placed at liquid nitrogen surface to freeze them. The blocks are conserved at -80°C.
Frozen lungs are cut into 5µm sections with a cryostat at -20°C and put on Super Frost + slides (Thermo Scientific, ref: 4951PLUS). Cut an optimal of 10 slides per organs with a maximum of two organs per slides. Slides are conserved at -20°C.

Slides are hydrated with fresh PBS for 10 minutes. Make a pool of PBS on the slide to prevent the organ to dry. Non-specific sites are blocked by adding 200 µl of PBS-BR 1% on each slides. Incubate for 20min at RT. During the incubation time, dilute the appropriate combination of markers/antibodies in PBS-BR 1% and keep them at 4°C. At the end of the incubation time, wash several times the slides in fresh PBS. Make a pool of PBS on the slides. Add 200µl of diluted markers/antibodies and incubate overnight at 4°C in a humid box. The next day, wash the slide twenty times in 3 successive pool of fresh PBS.

Make a pool of PBS on the slides. Add 200µl of secondary antibodies diluted in PBS-BR 1%.

Markers & Antibodies	Chanel	Dilution	Manufacturer	Reference
DAPI	Alexa Fluor 355	1/1000	Sigma-Aldrich	D9542
Phalloidine	Alexa Fluor 488	1/100	Invitrogen	A12379
CD11b	Alexa Fluor 647	1/200	eBioscience	51-0114-82
CD11c	Alexa Fluor 647 (APC)	1/100	BD Biosciences	550261
CD90.2	Alexa Fluor 647	1/200	Biolegend	105317
MHCII	Alexa Fluor 647 (APC)	1/100	eBioscience	17-5321-82
F4/80	Alexa Fluor 647 (APC)	1/100	eBioscience	17-4801-82
Ly-6G GR1	Alexa Fluor 647	1/200	eBioscience	51-5931-82
Anti LPS Brucella (from rabbit)	Biotin	1/500	UNamur	

Incubate for 2h at 4°C. At the end of the incubation time, wash the slide twenty times in 3 successive pool of fresh PBS.

Assemble the cover slip on the slide with FluoroGel medium (Electron microscopy Sciences - ref: 1785-10). Observation of the slides is realized with an inverted fluorescent microscope (Axiovert 200 Zeiss) and pictures are captured with a monochromic camera (Axiocam HR Zeiss). The analyses were performed using the AxioVision Rel 4.8 software.

Confocal microscopy

Observation of the slides is realized with an inverted fluorescent microscope (Observer Z1 Zeiss) with an oil immersion objective (100x, Plan Apo) and pictures are captured with a monochromic camera (Axiocam HR Zeiss). Picture for each fluorochrome were captured every 1µm stack of the 10µm lung section then merged and three dimension reconstruction was processed by the Imaris software. The analyses were performed using the Zen software.

Reagents.

Paraformaldehyde (PFA) 4%: Fixating agent

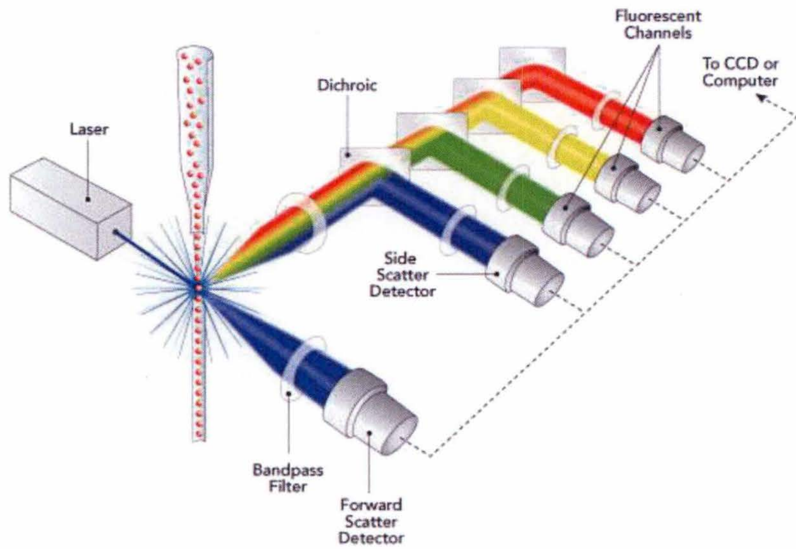
4g of PFA dissolved in 50mL of bidistillated water by agitation, heating up to 70°C and by adding drops of NaOH 1M until dissolution. The solution is cooled down on ice. 10mL of PBS 10x added and put to pH 7,4. The operation is done under chemical safety cabinet. The solution is fulfilled with bidistillated water up to 100mL and filtered with a 0.22µm filter. PFA is stored at -20°C. Can not be frozen again once unfrozen.

PBS-BR 1%: Non specific site blocking agent

Blocking Reagent (BR) dissolved in PBS by agitation and heating up to 60°C. Putted to pH 7,4. Can be conserved up to one week at 4°C. Blocking Reagent (Roche – ref: 11096176001)

Cell treatment and staining for FACS

FACS is a useful tool to characterize, in our case, the recruitment of immune cells and their related production of cytokines in an organ. FACS is based on the conjunction of a fluidic, an optical and an electronic system. The fluidic system induce a laminar flow of individual cells. These cells pass through the lasers beam. The light diffracted by the cells gives the information on the size of the cells (Forward Scatter, FSC). The reflexion and the refraction of the light by the cell are detected perpendicularly to the beam and indicate the relative granularity of the cell (Side Scatter, SSC). Precise determination of a cell type is done by a combination of specific antibodies coupled with different fluorochromes. These antibodies can bind on the surface of the cell or inside it if the cell has been fixed and permeabilized. 3 different fluorochromes has been used: Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) and Allophycicyanin (APC). They are characterized by different exitation and emission wave lenght. The light signal emited by the fluorochrome once hit by the laser beam, is detected and transformed into an electric signal. This electric signal is then amplified by photomultiplicators. For analysis, cells are gated to discard autofluorescent cells. (Arnould, 2012)



Procedure.

Mice are euthanized by cervical dislocation and lungs are taken. Lungs are placed in 6 wells plate. Add 2 drops of CollagenaseD/DNAse mix (Collagenase [4000U/mL], Roche + DNAse [10mg/mL], Sigma in RPMI) to keep the organs humid. Shear the organ with scissors. Add 1ml of Collagenase/DNAse mix to each well. Separate aggregates. Incubate 1h at 37°C + 5% CO_2 with incline. Add 6 to 8mL of cold RPMI per well. Using a 1mL syringe, suck up and discharge the content of the well twenty times. Filter using a bridal veil (70µm) and collect in a falcon 15mL. Mind to detach the cells at the bottom of the wells. Centrifuge at 1400rpm 4°C for 7min.

Resuspend in Completed RPMI (5mL) + BD Golgi Stop, protein inhibitor transporter (1/1000, BD Biosciences - ref: 51-2092KZ). Unscrew the cap of the falcon. Incubate for 4H30 at 37°C + 5% CO_2. Centrifuge 7min at 1400rpm 4°C. Resuspend in completed RPMI (1mL). Keep at 4°C over night. *

Chanel	**Antibodies**	Dilution	Manufacturer	Reference
FITC	CD11b	1/200	BD Pharmigen	553310
	CD3e	1/200	BD Pharmigen	553062
	Ly-6G	1/400	BD Pharmigen	551460
PE	CD4	1/200	BD Pharmigen	553049
	CD8a	1/200	BD Biosciences	553033
	NK1.1	1/200	BD Biosciences	553165
	CD11c	1/200	BD Pharmigen	553802
	Ly-6G	1/400	BD Pharmigen	551461
	γδ TCR	1/200	BD Pharmigen	553178
APC	CD4	1/400	BD Biosciences	553051
	IFNγ	1/300	BD Pharmigen	554413
	IL17a	1/300	eBioscience	17-7177-81
	CD11b	1/200	BD Biosciences	553312
	F4/80	1/200	eBioscience	17-4801-82
	Siglec-F	1/200	BD Pharmigen	55126
	MHCII	1/800	eBioscience	17-5321-81
-	24G2 (CD16/CD32)	1/200	BD Pharmigen	553142

Usual combination for cell phenotyping.

Cell phenotype	**Markers combination**
Alveolar Macrophages	F4/80^{+} CD11b^{med} CD11c^{high} Ly-6G^{-}
Interstitial Macrophages	F4/80^{+} CD11b^{med} CD11c^{-} Ly-6G^{-}
Neutrophils	F4/80^{-} CD11b^{high} CD11c^{-} Ly-6G^{high} SSC^{high}
Eosinophils	CD11b^{+} Ly-6G^{+} Siglec-F^{+} SSC^{high}
classical Dendritic Cells	CD11b^{high} CD11c ^{med} Ly-6G^{+}
inflammatory Dendritic Cells	CD11b^{high} CD11c ^{high} Ly-6G^{+}
Lymphocyte T CD4	TCRβ^{+} CD3^{+} CD4^{+}
γδ T cells	TCRγδ^{+} CD11b^{-} CD3^{+}

Statistical analysis. All data analysis were performed through the program GraphPad Prism. ELISA data are presented as means ±SDs. CFU data are presented as individual dots and means ±SDs. The differences between the means values were estimated by a Mann-Whitney test. In all figures * symbol is used to indicate significant differences between groups (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

Centrifuge 7min at 1400rpm 4°C. Throw away the supernatant and resuspend in FACS buffer (200µL). Add 150µL to a 96 wells plate. Centrifuge the plate 1min45" at 2000rpm and resuspend in FACS buffer + 24G2 (anti-Fc antibody). Keep at 4°C for 20min. Centrifuge the plate 1min45" at 2000rpm and resuspend with appropriate surface antibody mix diluted in cold FACS buffer. Keep at 4°C for 30min. Centrifuge the plate 1min45" at 2000rpm and renew the supernatant by FACS buffer. (3 times) Resuspend in PAF1% (150µL) diluted in FACS buffer. Keep at 4°C for 15min.
Centrifuge the plate 1min45" at 2000rpm and renew the supernatant by FACS buffer. (3 times) Resuspend in intracellular antibody mix (150µL) + Permwash (10%, BD Biosciences - ref: 51-2091KZ) diluted in FACS buffer. Keep at 4°C for 30min. Centrifuge the plate 1min45" at 2000rpm and renew the supernatant by FACS buffer. (3 times) *
Centrifuge the plate 1min45" at 2000rpm and renew the supernatant by FACS buffer. (3 times) Resuspend in FACS buffer (150µL). Transfer in FACS tube (1mL) containing already PAF1% (400µL).
*If intracellular staining

Medium. Completed RPMI, rich culture medium. RPMI (500mL) completed with 50mL of decomplemented (57°C, 30 minutes) FBS (Invitrogen, ref: 10270-106), 5mL of L-Glutamin (Life technologies, ref: 25030-024), 5mL of Non essential amino acids (Life technologies, ref: 11140-035), 5mL of Pyruvate sodium (Life technologies, ref: 11360-039) and gentamycin (50mg/mL). Keep at 4°C.

FACS Buffer. BSA [2g/L] and NaN_3 azide [0,2g/L] dissolved in PBS under agitation. Do not autoclave.

Reagents.

Collagenase (4000U/mL):

Resuspend lyophilized collagenase D in RPMI to obtain 4000U/mL and aliquote (1mL) in ependorf. (Collagenase D, Roche, ref : 11088866001)

$$\frac{\text{Enzymatic Activity (U)} \times 750\ (\text{mL}) \times 500\ (\text{mg})}{4000\ (\text{U/mg})} = \quad (\text{mL})$$

rem: non sterile

DNAse:

Resuspend lyophilized DNAse in autoclaved NaCl 0.15M solution to obtain a final concentration of 10mg/mL. Aliquote in ependorf (100µL). (Deoxyribonuclease I from bovine pancreas, SIGMA, cat : D4513)

REFERENCES

Akbari, O., Freeman, G.J., Meyer, E.H., Greenfield, E.A., Chang, T.T., Sharpe, A.H., Berry, G., DeKruyff, R.H., and Umetsu, D.T. (2002). Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat. Med. *8*, 1024–1032.

Apiwattanakul, N., Thomas, P.G., Iverson, A.R., and McCullers, J.A. (2014). Chronic helminth infections impair pneumococcal vaccine responses. Vaccine 5405–5410.

Archambaud, C., Salcedo, S.P., Lelouard, H., Devilard, E., de Bovis, B., Van Rooijen, N., Gorvel, J.-P., and Malissen, B. (2010). Contrasting roles of macrophages and dendritic cells in controlling initial pulmonary Brucella infection. Eur. J. Immunol. *40*, 3458–3471.

Arnold, I.C., Dehzad, N., Reuter, S., Martin, H., Becher, B., Taube, C., and Müller, A. (2011). Brief report Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. J. Clin. Invest. *121*, 3088–3093.

Arora, M., Poe, S.L., Oriss, T.B., Krishnamoorthy, N., Yarlagadda, M., Wenzel, S.E., Billiar, T.R., Ray, a, and Ray, P. (2010). TLR4/MyD88-induced CD11b+Gr-1 int F4/80+ non-migratory myeloid cells suppress Th2 effector function in the lung. Mucosal Immunol. *3*, 578–593.

Atluri, V.L., Xavier, M.N., de Jong, M.F., den Hartigh, A.B., and Tsolis, R.M. (2011). Interactions of the human pathogenic Brucella species with their hosts. Annu. Rev. Microbiol. *65*, 523–541.

Baldwin, C.L., and Goenka, R. (2006). Host Immune Responses to the Intracellular Bacteria Brucella: Does the Bacteria Instruct the Host to Facilitate Chronic Infection? Crit. Rev. Immunol. *26*, 407–442.

Baldwin, C.L., Jiang, X., and Fernandes, D.M. (1993). Macrophage control of Brucella abortus: Influence of cytokines and iron. Trends Microbiol. *1*, 99–104.

Banatvala, N., Mayo, K., Megraud, F., Jennings, R., Deeks, J.J., and Feldman, R.A. (1993). The cohort effect and Helicobacter pylori. J. Infect. Dis. *168*, 219–221.

Barnes, P.J. (2008). Immunology of asthma and chronic obstructive pulmonary disease. Nat. Rev. Immunol. *8*, 183–192.

Barquero-Calvo, E., Martirosyan, A., Ordoñez-Rueda, D., Arce-Gorvel, V., Alfaro-Alarcón, A., Lepidi, H., Malissen, B., Malissen, M., Gorvel, J.-P., and Moreno, E. (2013). Neutrophils exert a suppressive effect on Th1 responses to intracellular pathogen Brucella abortus. PLoS Pathog. *9*, e1003167.

Bates, J.H.T., Rincon, M., and Irvin, C.G. (2009). Animal models of asthma. Am. J. Physiol. Lung Cell. Mol. Physiol. *297*, L401–L410.

Bedoret, D., Wallemacq, H., Marichal, T., Desmet, C., Calvo, F.Q., Henry, E., Closset, R., Dewals, B., Thielen, C., Gustin, P., et al. (2009). Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *119*.

Blaser, M.J., and Falkow, S. (2009). What are the consequences of the disappearing human microbiota? Nat. Rev. Microbiol. *7*, 887–894.

Braman, S.S. (2006). The global burden of asthma. In Chest,.

Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., and Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat. Immunol. 1–11.

Brinkmann, V., and Zychlinsky, A. (2007). Beneficial suicide : why neutrophils. Nat. Rev. Microbiol. *5*, 577–582.

Brumell, J.H. (2012). Brucella "hitches a ride" with autophagy. Cell Host Microbe *11*, 2–4.

Castañeda-Roldán, E.I., Ouahrani-Bettache, S., Saldaña, Z., Avelino, F., Rendón, M. a, Dornand, J., and Girón, J. a (2006). Characterization of SP41, a surface protein of Brucella associated with adherence and invasion of host epithelial cells. Cell. Microbiol. *8*, 1877–1887.

Chen, F., Liu, Z., Wu, W., Rozo, C., Bowdridge, S., Millman, A., Van Rooijen, N., Urban, J.F., Wynn, T. a, and Gause, W.C. (2012). An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. Nat. Med. *18*, 260–266.

Conde-Álvarez, R., Arce-Gorvel, V., Iriarte, M., Manček-Keber, M., Barquero-Calvo, E., Palacios-Chaves, L., Chacón-Díaz, C., Chaves-Olarte, E., Martirosyan, A., von Bargen, K., et al. (2012). The lipopolysaccharide core of Brucella abortus acts as a shield against innate immunity recognition. PLoS Pathog. *8*, e1002675.

Copin, R., Baetselier, P. De, Carlier, Y., Letesson, J., and Muraille, E. (2007). MyD88-Dependent Activation of B220 ٪.. CD11b ٪. LY-6C ٪. Dendritic Cells during. J. Immunol. *178*, 5182–5191.

Copin, R., Vitry, M.-A., Hanot Mambres, D., Machelart, A., De Trez, C., Vanderwinden, J.-M., Magez, S., Akira, S., Ryffel, B., Carlier, Y., et al. (2012). In situ microscopy analysis reveals local innate immune response developed around Brucella infected cells in resistant and susceptible mice. PLoS Pathog. *8*, e1002575.

Cua, D.J., and Tato, C.M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. Nat. Rev. Immunol. *10*, 479–489.

Denis, O., van den Brûle, S., Heymans, J., Havaux, X., Rochard, C., Huaux, F., and Huygen, K. (2007). Chronic intranasal administration of mould spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodelling. Immunology *122*, 268–278.

Devereux, G. (2006). The increase in the prevalence of asthma and allergy: food for thought. Nat. Rev. Immunol. *6*, 869–874.

Diebold, S.S. (2008). Determination of T-cell fate by dendritic cells. Immunol. Cell Biol. *86*, 389–397.

Donnelly, S., Dalton, J.P., and Loukas, A. (2006). Proteases in helminth- and allergen-induced inflammatory responses. Chem. Immunol. Allergy *90*, 45–64.

Durward, M., Radhakrishnan, G., Harms, J., Bareiss, C., Magnani, D., and Splitter, G.A. (2012). Active Evasion of CTL Mediated Killing and Low Quality Responding CD8+ T Cells Contribute to Persistence of Brucellosis. PLoS One *7*, e34925.

Eash, K.J., Greenbaum, A.M., Gopalan, P.K., and Link, D.C. (2010). CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. J. Clin. Invest. *120*, 2423–2431.

Edwards, M.R., Bartlett, N.W., Hussell, T., Openshaw, P., and Johnston, S.L. (2012). The microbiology of asthma. Nat. Rev. Microbiol. *10*, 459–471.

Elias, D., Akuffo, H., and Britton, S. (2006a). Helminthes could influence the outcome of vaccines against TB in the tropics. Parasite Immunol. *28*, 507–513.

Elias, D., Akuffo, H., and Britton, S. (2006b). Helminthes could influence the outcome of vaccines against TB in the tropics. Parasite Immunol. *28*, 507–513.

Elo, L.L., Järvenpää, H., Tuomela, S., Raghav, S., Ahlfors, H., Laurila, K., Gupta, B., Lund, R.J., Tahvanainen, J., Hawkins, R.D., et al. (2010). Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. Immunity *32*, 852–862.

Ernst, J.D. (2012). The immunological life cycle of tuberculosis. Nat. Rev. Immunol. *12*, 581–591.

Felton, J.M., Lucas, C.D., Rossi, A.G., and Dransfield, I. (2014). Eosinophils in the lung – modulating apoptosis and efferocytosis in airway inflammation. *5*, 1–11.

Ferretti, S., Bonneau, O., Dubois, G.R., Jones, C.E., and Trifilieff, A. (2003). IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J. Immunol. *170*, 2106–2112.

Forestier, C., Moreno, E., Pizarro-Cerda, J., and Gorvel, J.P. (1999). Lysosomal accumulation and recycling of lipopolysaccharide to the cell surface of murine macrophages, an in vitro and in vivo study. J. Immunol. *162*, 6784–6791.

Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., and Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. J. Cell Biol. *176*, 231–241.

Gaffen, S.L., Jain, R., Garg, A. V., and Cua, D.J. (2014). The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. Nat. Rev. Immunol. *14*, 585–600.

Godfroid, J., Scholz, H.C., Barbier, T., Nicolas, C., Wattiau, P., Fretin, D., Whatmore, a M., Cloeckaert, a, Blasco, J.M., Moriyon, I., et al. (2011). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. Prev. Vet. Med. *102*, 118–131.

Goenka, R., Parent, M.A., Elzer, P.H., and Baldwin, C.L. (2011). B cell-deficient mice display markedly enhanced resistance to the intracellular bacterium Brucella abortus. J. Infect. Dis. *203*, 1136–1146.

Gorvel, L., Textoris, J., Banchereau, R., Ben Amara, A., Tantibhedhyangkul, W., von Bargen, K., Ka, M.B., Capo, C., Ghigo, E., Gorvel, J.-P., et al. (2014). Intracellular bacteria interfere with dendritic cell functions: role of the type I interferon pathway. PLoS One *9*, e99420.

Guilliams, M., Lambrecht, B.N., and Hammad, H. (2013). Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. Mucosal Immunol. *6*, 464–473.

Habibzay, M., Saldana, J.I., Goulding, J., Lloyd, C.M., and Hussell, T. (2012). Altered regulation of Toll – like receptor responses impairs antibacterial immunity in the allergic lung. Mucosal Immunol. *5*, 524–534.

Habibzay, M., Weiss, G., and Hussell, T. (2013). Bacterial superinfection following lung inflammatory disorders. Future Microbiol. *8*, 247–256.

Hammad, H., Chieppa, M., Perros, F., Willart, M.A., Germain, R.N., and Lambrecht, B.N. (2009). House dust mite allergen induces asthma via TLR4 triggering of airway structural cells. Nat. Med. *15*, 410–416.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., Davies, J., Ervine, A., Poulter, L., Pachter, L., et al. (2010). Disordered microbial communities in asthmatic airways. PLoS One *5*.

Holgate, S.T. (2012). Innate and adaptive immune responses in asthma. Nat. Med. *18*, 673–683.

Holgate, S.T., and Davies, D.E. (2009). Rethinking the pathogenesis of asthma. Immunity *31*, 362–367.

Holgate, S.T., and Polosa, R. (2008). Treatment strategies for allergy and asthma. Nat. Rev. Immunol. *8*, 218–230.

Holt, P.G., Oliver, J., Bilyk, N., McMenamin, C., McMenamin, P.G., Kraal, G., and Thepen, T. (1993). Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. J. Exp. Med. *177*, 397–407.

Holt, P.G., Strickland, D.H., Wikström, M.E., and Jahnsen, F.L. (2008). Regulation of immunological homeostasis in the respiratory tract. Nat. Rev. Immunol. *8*, 142–152.

Hussell, T., and Bell, T.J. (2014). Alveolar macrophages: plasticity in a tissue-specific context. Nat. Rev. Immunol. *14*, 81–93.

Iannino, F., Ugalde, J.E., and Iñón de Iannino, N. (2012). Brucella abortus efp gene is required for an efficient internalization in HeLa cells. Microb. Pathog. *52*, 31–40.

Ibañez, A.E., Smaldini, P., Coria, L.M., Delpino, M. V, Pacífico, L.G.G., Oliveira, S.C., Risso, G.S., Pasquevich, K. a, Fossati, C.A., Giambartolomei, G.H., et al. (2013). Unlipidated outer membrane protein Omp16 (U-Omp16) from Brucella spp. as nasal adjuvant induces a Th1 immune response and modulates the Th2 allergic response to cow's milk proteins. PLoS One *8*, e69438.

Jakubzick, C., Tacke, F., Llodra, J., van Rooijen, N., and Randolph, G.J. (2006). Modulation of dendritic cell trafficking to and from the airways. J. Immunol. *176*, 3578–3584.

Johnston, S.L., Blasi, F., Black, P.N., Martin, R.J., Farrell, D.J., and Nieman, R.B. (2006). The effect of telithromycin in acute exacerbations of asthma. N. Engl. J. Med. *354*, 1589–1600.

Kaufmann, S.H.E. (2007). The contribution of immunology to the rational design of novel antibacterial vaccines. Nat. Rev. Microbiol. *5*, 491–504.

Ko, J., Gendron-Fitzpatrick, A., and Splitter, G.A. (2002). Susceptibility of IFN regulatory factor-1 and IFN consensus sequence binding protein-deficient mice to brucellosis. J. Immunol. *168*, 2433–2440.

Kreutzer, D.L., Dreyfus, L.A., and Robertson, D.C. (1979). Interaction of polymorphonuclear leukocytes with smooth and rough strains of Brucella abortus. Infect. Immun. *23*, 737–742.

Kusel, M.M.H., de Klerk, N.H., Kebadze, T., Vohma, V., Holt, P.G., Johnston, S.L., and Sly, P.D. (2007). Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J. Allergy Clin. Immunol. *119*, 1105–1110.

Lacerda, T.L.S., Salcedo, S.P., and Gorvel, J.-P. (2013). Brucella T4SS: the VIP pass inside host cells. Curr. Opin. Microbiol. *16*, 45–51.

Lambrecht, B.N., and Hammad, H. (2003). Taking our breath away: dendritic cells in the pathogenesis of asthma. *3*, 994–1003.

Lambrecht, B.N., and Hammad, H. (2009). Biology of lung dendritic cells at the origin of asthma. Immunity *31*, 412–424.

Lapaque, N., Forquet, F., de Chastellier, C., Mishal, Z., Jolly, G., Moreno, E., Moriyon, I., Heuser, J.E., He, H.-T., and Gorvel, J.-P. (2006). Characterization of Brucella abortus lipopolysaccharide macrodomains as mega rafts. Cell. Microbiol. *8*, 197–206.

Lauzon-Joset, J.-F., Marsolais, D., Langlois, a, and Bissonnette, E.Y. (2014). Dysregulation of alveolar macrophages unleashes dendritic cell-mediated mechanisms of allergic airway inflammation. Mucosal Immunol. *7*, 155–164.

Liew, F.Y. (2012). IL-33: a Janus cytokine. Ann. Rheum. Dis. *71 Suppl 2*, i101–i104.

Lloyd, C.M., and Murdoch, J.R. (2010). Tolerizing allergic responses in the lung. Mucosal Immunol. *3*, 334–344.

Mak, T., Saunders, M., and Jett, B. (2014). Primer to the immune response (Elsevier).

Marichal, T., Bedoret, D., Mesnil, C., Pichavant, M., Goriely, S., Trottein, F., Cataldo, D., Goldman, M., Lekeux, P., Bureau, F., et al. (2010). Interferon response factor 3 is essential for house dust mite-induced airway allergy. J. Allergy Clin. Immunol. *126*, 836–844.e13.

Marichal, T., Starkl, P., Reber, L.L., Kalesnikoff, J., Oettgen, H.C., Tsai, M., Metz, M., and Galli, S.J. (2013). A beneficial role for immunoglobulin E in host defense against honeybee venom. Immunity *39*, 963–975.

Martirosyan, A., and Gorvel, J. (2013). Brucella evasion of adaptive immunity. Future Microbiol. *8*, 147–154.

Martirosyan, A., and Rica, C. (2011). An evolutionary strategy for a stealthy intracellular Brucella pathogen. Immunol. Rev. *240*, 211–234.

Mathew, A., MacLean, J.A., DeHaan, E., Tager, A.M., Green, F.H., and Luster, A.D. (2001). Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation. J. Exp. Med. *193*, 1087–1096.

Maus, U.A., Janzen, S., Wall, G., Srivastava, M., Blackwell, T.S., Christman, J.W., Seeger, W., Welte, T., and Lohmeyer, J. (2006). Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. Am. J. Respir. Cell Mol. Biol. *35*, 227–235.

McCullers, J. a (2014). The co-pathogenesis of influenza viruses with bacteria in the lung. Nat. Rev. Microbiol. *12*, 252–262.

McSorley, H.J., Hewitson, J.P., and Maizels, R.M. (2013). Immunomodulation by helminth parasites: defining mechanisms and mediators. Int. J. Parasitol. *43*, 301–310.

Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. *7*, 145–173.

Muraille, E., Leo, O., and Moser, M. (2014). Th1/Th2 paradigm extended: Macrophage polarization as an unappreciated pathogen-driven escape mechanism? Name Front. Immunol. *5*, 1–12.

Murphy, E.A., Sathiyaseelan, J., Parent, M.A., Zou, B., and Baldwin, C.L. (2001). Interferon-?? is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology *103*, 511–518.

Nathan, A.T., Peterson, E.A., Chakir, J., and Wills-Karp, M. (2009). Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. J. Allergy Clin. Immunol. *123*, 612–618.

O'Shea, J.J., Lahesmaa, R., Vahedi, G., Laurence, A., and Kanno, Y. (2011). Genomic views of STAT function in CD4+ T helper cell differentiation. Nat. Rev. Immunol. *11*, 239–250.

Ouyang, W., Kolls, J.K., and Zheng, Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity *28*, 454–467.

Palm, N.W., Rosenstein, R.K., and Medzhitov, R. (2012). Allergic host defences. Nature *484*, 465–472.

Palm, N.W., Rosenstein, R.K., Yu, S., Schenten, D.D., Florsheim, E., and Medzhitov, R. (2013). Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. Immunity *39*, 976–985.

Pappas, G., Papadimitriou, P., Akritidis, N., Christou, L., and Tsianos, E. V (2006). The new global map of human brucellosis. Lancet Infect. Dis. *6*, 91–99.

Paul, W.E., and Zhu, J. (2012). How are TH2-type immune responses initiated and amplified. Nat. Rev. Immunol. *10*, 225–235.

Peng, M.Y., Wang, Z.H., Yao, C.Y., Jiang, L.N., Jin, Q.L., Wang, J., and Li, B.Q. (2008). Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. Cell. Mol. Immunol. *5*, 203–208.

Potian, J. a, Rafi, W., Bhatt, K., McBride, A., Gause, W.C., and Salgame, P. (2011). Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. J. Exp. Med. *208*, 1863–1874.

Profet, M. (1991). The function of allergy: immunological defense against toxins. Q. Rev. Biol. *66*, 23–62.

Rajashekara, G., Glover, D.A., Krepps, M., and Splitter, G.A. (2005). Temporal analysis of pathogenic events in virulent and avirulent Brucella melitensis infections. Cell. Microbiol. *7*, 1459–1473.

Van Riet, E., Hartgers, F.C., and Yazdanbakhsh, M. (2007). Chronic helminth infections induce immunomodulation: Consequences and mechanisms. Immunobiology *212*, 475–490.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. *2*, 845–858.

Salcedo, S.P., Marchesini, M.I., Degos, C., Terwagne, M., Von Bargen, K., Lepidi, H., Herrmann, C.K., Santos Lacerda, T.L., Imbert, P.R.C., Pierre, P., et al. (2013). BtpB, a novel Brucella TIR-containing effector protein with immune modulatory functions. Front. Cell. Infect. Microbiol. *3*, 28.

Sathiyaseelan, J., Jiang, X., and Baldwin, C.L. (2000). Growth of Brucella abortus in macrophages from resistant and susceptible mouse strains. Clin. Exp. Immunol. *121*, 289–294.

Smart, S.J., and Casale, T.B. (1994). Pulmonary epithelial cells facilitate TNF-alpha-induced neutrophil chemotaxis. A role for cytokine networking. J. Immunol. *152*, 4087–4094.

Starr, T., Ng, T.W., Wehrly, T.D., Knodler, L. a, and Celli, J. (2008). Brucella intracellular replication requires trafficking through the late endosomal/lysosomal compartment. Traffic *9*, 678–694.

Starr, T., Child, R., Wehrly, T.D., Hansen, B., Hwang, S., López-Otin, C., Virgin, H.W., and Celli, J. (2012). Selective subversion of autophagy complexes facilitates completion of the Brucella intracellular cycle. Cell Host Microbe *11*, 33–45.

Stelekati, E., and Wherry, E.J. (2012). Chronic bystander infections and immunity to unrelated antigens. Cell Host Microbe *12*, 458–469.

Strachan, D.P. (1989). Hay fever, hygiene, and household size. BMJ *299*, 1259–1260.

Swain, S.L., McKinstry, K.K., and Strutt, T.M. (2012). Expanding roles for $CD4^+$ T cells in immunity to viruses. Nat. Rev. Immunol. *12*, 136–148.

Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R.S., Madan, R., Thorne, P.S., Wills-Karp, M., Gioannini, T.L., Weiss, J.P., et al. (2009). Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature *457*, 585–588.

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of γδ T cells to immunology. Nat. Rev. Immunol. *13*, 88–100.

Vercelli, D. (2008). Discovering susceptibility genes for asthma and allergy. Nat. Rev. Immunol. *8*, 169–182.

Vitry, M.-A., De Trez, C., Goriely, S., Dumoutier, L., Akira, S., Ryffel, B., Carlier, Y., Letesson, J.-J., and Muraille, E. (2012). Crucial role of gamma interferon-producing CD4+ Th1 cells but dispensable function of CD8+ T cell, B cell, Th2, and Th17 responses in the control of Brucella melitensis infection in mice. Infect. Immun. *80*, 4271–4280.

Vitry, M.-A., Hanot Mambres, D., Deghelt, M., Hack, K., Machelart, A., Lhomme, F., Vanderwinden, J.-M., Vermeersch, M., De Trez, C., Pérez-Morga, D., et al. (2014a). Brucella melitensis invades murine erythrocytes during infection. Infect. Immun. *82*, 3927–3938.

Vitry, M.-A., Hanot Mambres, D., De Trez, C., Akira, S., Ryffel, B., Letesson, J.-J., and Muraille, E. (2014b). Humoral immunity and CD4+ Th1 cells are both necessary for a fully protective immune response upon secondary infection with Brucella melitensis. J. Immunol. *192*, 3740–3752.

Wakashin, H., Hirose, K., Maezawa, Y., Kagami, S.I., Suto, A., Watanabe, N., Saito, Y., Hatano, M., Tokuhisa, T., Iwakura, Y., et al. (2008). IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. Am. J. Respir. Crit. Care Med. *178*, 1023–1032.

Wenzel, S.E. (2012). Asthma phenotypes: the evolution from clinical to molecular approaches. Nat. Med. *18*, 716–725.

Wernersson, S., and Pejler, G. (2014). Mast cell secretory granules: armed for battle. Nat. Rev. Immunol. *14*, 478–494.

Williams, A. (2012). Immunology: Mucosal and Body Surface Defences (Chichester, West Sussex: Wiley-Blackwell).

Wilson, R., Dowling, R.B., and Jackson, a. D. (1996). The biology of bacterial colonization and invasion of the respiratory mucosa. Eur. Respir. J. *9*, 1523–1530.

Wu, L.C., and Zarrin, A. a (2014). The production and regulation of IgE by the immune system. Nat. Rev. Immunol. *14*, 247–259.

Xavier, M.N., Paixão, T.A., Hartigh, A.B. Den, Tsolis, R.M., and Santos, R.L. (2010). Pathogenesis of Brucella spp . 109–118.

Xavier, M.N., Winter, M.G., Spees, A.M., den Hartigh, A.B., Nguyen, K., Roux, C.M., Silva, T.M. a, Atluri, V.L., Kerrinnes, T., Keestra, a M., et al. (2013a). PPARγ-mediated increase in glucose availability sustains chronic Brucella abortus infection in alternatively activated macrophages. Cell Host Microbe *14*, 159–170.

Xavier, M.N., Winter, M.G., Spees, A.M., Nguyen, K., Atluri, V.L., Silva, T.M. a, Bäumler, A.J., Müller, W., Santos, R.L., and Tsolis, R.M. (2013b). CD4+ T cell-derived IL-10 promotes Brucella abortus persistence via modulation of macrophage function. PLoS Pathog. *9*, e1003454.

Young, B., Lowe, J.S., Stevens, A., and Heath, J.W. (2006). Wheater's Functional Histology: A Text and Colour Atlas (Elsevier).

Zwerdling, A., Delpino, M.V., Pasquevich, K. a, Barrionuevo, P., Cassataro, J., García Samartino, C., and Giambartolomei, G.H. (2009). Brucella abortus activates human neutrophils. Microbes Infect. *11*, 689–697.